OPTIMIZATION OF HEPATIC TARGETING FOR ANTISENSE INHIBITION OF HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS

By

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NOTATIONS

SHR	Spontaneously hypertensive rat
WKY	Wistar Kyoto rat
AGT	Angiotensinogen
RAS	Renin-angiotensin system
ASODN	Antisense oligodeoxynucleotide
PC:Cholesterol	Phosphatidylcholine: cholesterol
DDAB	Dimethyldioctadecylammonium bromide
DOPE	Dioleoylphosphatidylethanolamine
CA/ASODN	Cationic liposome-complexed ASODN
CA/ScrODN	Cationic liposome-complexed scrambled oligonucleotide
PC/ASODN	PC:cholesterol liposome-encapsulated ASODN
FITC-ASODN	Fluorescein isothiocyanate-conjugated ASODN

Abstract of A Dissertation presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Chairman: Donna Wielbo, Ph.D.
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The role of angiotensinogen (AGT) in the pathogenesis of hypertension, a major risk to human health, is well supported. Scientific studies have previously shown that the blockade of angiotensinogen gene expression has been effective in decreasing blood pressure. This dissertation focuses on the effect of cationic liposomes (CA) as a delivery system for an antisense oligodeoxynucleotide (ASODN) targeted to the AGT gene in rat hepatoma cell culture and in the spontaneously hypertensive rat (SHR) model of hypertension. The pharmacological effects of cationic liposome-complexed ASODN (CA/ASODN) on AGT mRNA and protein expression, blood pressure, and both hormonal and tissue renin-angiotensin system (RAS) in SHR are studied. The results presented demonstrate that cationic

liposome increases cellular uptake and tissue distribution of ASODN. An increased cellular uptake of ASODN resulted in enhanced gene inhibition and biological effects. These effects were demonstrated by a dose-dependent decrease in AGT mRNA and protein in hepatoma cell culture and decreased blood pressure, plasma AGT, plasma Angiotensin II and AGT mRNA in heart, kidney, and liver in the SHR. Decreases in blood pressure were also shown to correlate with decreases in AGT mRNA in the kidney, suggesting renally mediated mechanisms may contribute to the observed blood pressure decrease. Plasma aldosterone levels also decreased after CA/ASODN treatment with a concomitant increase in urine output, a decrease in urinary potassium excretion and an increase in sodium excretion. These studies strongly suggest that ASODN targeted to AGT mRNA has the potential to be used as a therapeutic agent for the treatment of hypertension as well as a research tool to study the mechanisms of development and maintenance of hypertension.

CHAPTER 1
INTRODUCTION

Hypertension

Overview

Approximately 20% of the adult population in the United States suffers from hypertension[1]. Untreated, sustained hypertension can lead to serious complications such as congestive heart failure, myocardial infarction, and cerebrovascular hemorrhage[2], making it a major threat to human life. Hypertension is defined as an abnormal elevation of systemic arterial blood pressure. In a healthy human, blood pressure levels fluctuate within certain limits depending on body position, age, and stress. Adult individuals with blood pressure measurements in excess of 140/90 mm Hg are considered hypertensive. Ninety five percent of all hypertension has no identifiable cause and is termed essential or primary hypertension. Hypertension that results from other known diseases, is termed secondary hypertension. The most common cause of secondary hypertension is renovascular disease, such as acute and chronic glomerulonephritis. Cushing's disease, primary aldosteronism, pheochromocytoma, and coarctation of the

aorta are other causes of secondary hypertension[2].

Blood pressure homeostasis is maintained by the pumping action of the heart (cardiac output), the volume of the vascular system, and the tone of the peripheral vasculature (peripheral resistance). Blood pressure is determined by the product of cardiac output and peripheral resistance[3]. Three major mechanisms are responsible for normal blood pressure regulation: the fast neural baroreceptor feedback mechanism, the slower endocrine renin-angiotensin aldosterone system, and the renal regulation of water and sodium homeostasis[3].In the pathophysiological hypertensive condition, cardiac output and peripheral resistance deviate from the normal range via the interaction of a complex series of factors. Mechanisms currently being investigated include pressor and depressor factors of renal origin, neurogenic regulation, circulating humoral factors, vessel wall hypertrophy, and membrane transport abnormality[4].

The interdependence of different systems on hypertension is described in Figure 1-1[1]. As shown, increased cardiac output can result from increased cardiac contractility or increased fluid volume. Increased fluid volume mainly results from a renal defect, such as decreased renal filtration, which leads to increased renal sodium retention. Increased cardiac contractility is mostly a direct consequence of overactivity of the sympathetic nervous system. Increased peripheral resistance may result from functional

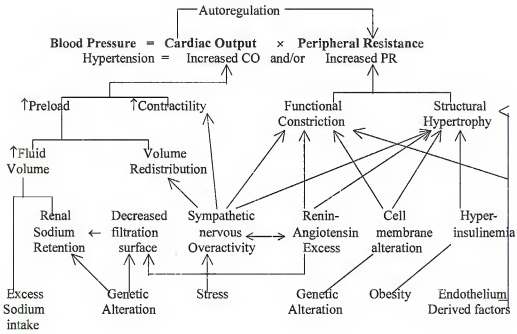


Figure 1-1. Factors involved in the control of blood pressure that affect the basic equation: blood pressure = cardiac output × peripheral resistance (adapted from reference 1).

constriction and structural hypertrophy of blood vessels. Among these factors, renin angiotensin system overactivity may lead to an increase in both cardiac output and peripheral resistance.

Among the different initiating factors of essential hypertension, the primacy of the kidney is supported by numerous studies[5]. For example, in experimental renal transplantation studies with rats, kidneys from genetically hypertensive donors, such as spontaneously hypertensive rats, consistently elicited hypertension in genetically normotensive recipients. Additionally, the removal of both native kidneys and transplantation of renal grafts from

genetically normotensive donors resulted in blood pressure normalization in genetically hypertensive rats, suggesting that renal mechanisms play a major role in the pathogenesis of hypertension in these strains[6].

Role of the Renin Angiotensin System (RAS) in Hypertension

The role of the renin angiotensin system (RAS) in the development and maintenance of hypertension is well established. The RAS plays a critical role in the control of blood pressure, fluid and eletrolyte homeostasis, and renal hemodynamics[7,8]. The primary components of the RAS include: (a) angiotensinogen, a 452 amino acid protein that serves as the substrate for (b) renin, the enzyme that catalyzes the proteolytic conversion of angiotensinogen to the decapeptide angiotensin I; (c) angiotensin converting enzyme, a dipeptidyl carboxypeptidase that converts angiotensin I to the octapeptide angiotensin II; (d) angiotensin II itself; and (e) the angiotensin II receptor which is responsible for transducing the cellular effects of angiotensin II (Figure 1- 2)[1]. The binding of angiotensin II to its receptor mediates various physiological responses such as vasoconstriction, cell proliferation, aldosterone and vasopressin release and dipsogenesis[9,10]. Historically, this hormonal system has been viewed as an endocrine system. The various components of this cascade being derived from different organs which are then delivered to their sites of action by the circulatory system[9]. However, in

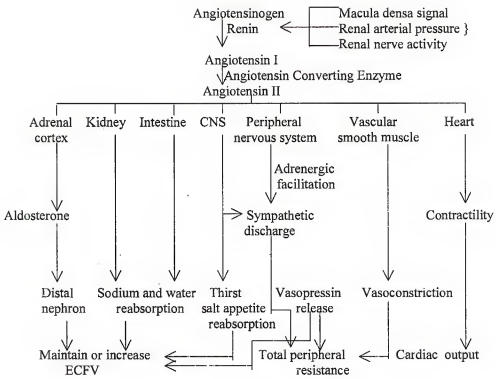


Figure 1-2. Schematic representation of the renin-angiotensin-system. CNS: central nervous system; ECFV: extracellular fluid volume (adapted from reference 1).

recent years there has been an increasing number of studies to suggest that in addition to this traditional hormonal RAS, there is a tissue RAS which produces II for local needs [11-16]. The evidence for a functional tissue RAS is based on a number of findings: (a) the demonstration of renin-like activity in extra-renal tissues such as brain, heart, adrenal, and blood vessels, suggesting local synthesis of Angiotensin II; (b) chronic treatment with angiotensin converting enzyme inhibitors fails to correlate with pretreatment plasma renin activity but does correlate with sustained inhibition of converting enzyme activity in the kidney; (c) ligand binding studies reveal that angiotensin

receptors are present in various tissues, indicating that angiotensin exerts organ-specific effects; (d) expression analysis of renin, angiotensinogen, and converting enzyme show that they are coexpressed in several tissues, including the kidneys, the brain, the anterior pituitary gland, the ovaries, the adrenal cortex, vascular smooth muscle, and the heart. This observation provides the basis for de novo synthesis of angiotensin II[14]. The existence of a local RAS also was supported by quantitative studies. For example, angiotensin II, the active peptide, has been shown to have an affinity for its receptor in the nanomolar range, but circulates at plasma levels in the picomolar range. This suggests a local angiotensin II-concentrating process in the vicinity of its receptor, and that plasma angiotensin II is not entirely responsible for the effects of the system[11]. The presence of a tissue RAS has been interpreted in paracrine/autocrine vs. endocrine models[17-19] which suggests that circulating endocrine angiotensin II participates in short-term mechanism of blood pressure and body fluid control, such as in the case of hemorrhage and aggressive diuretic treatment. While local paracrine/autocrine angiotensin II systems participate more in long term regulation and in angiotensin II dependent structural changes, the role of angiotensin II as a mitogenic, proliferative factor which contributes to vascular and cardiac hypertrophy is mediated in a paracrine manner. The tissue RAS may also serve as a mechanism to compliment or interact

with the hormonal RAS[12].

Renin

Renin, an aspartyl protease responsible for the first step in the formation of angiotensin II, is highly specific for its substrate angiotensinogen. The main source of renin is the juxtaglomerular cells of the afferent arterioles of the kidney. Renin levels are controlled at several stages, including the level of gene expression, rates of intracellular synthesis/processing, and the rate of secretion [20]. Translation of renin mRNA yields preprorenin, which then undergoes cotranslational removal of a single peptide and glycosylation during transport through the rough endoplasmic reticulum to become prorenin. Prorenin can either be constitutively secreted from the Golgi apparatus or packaged in immature granules and secreted in a regulated fashion. Although prorenin is the major circulating form of renin, the primary site of conversion of plasma prorenin to renin still remains unclear. Prorenin activating enzymes have been found in the kidney as well as in endothelial cells and neutrophils. In transgenic mice expressing the human renin gene, renin mRNA expression was found to be high in the kidney, adrenal, ovary, testis, lung, and adipose tissue and low in the heart and submandibular gland[21].

Expression of renin mRNA in various tissues appears to be differentially regulated. Sodium depletion or β-adrenergic receptor activation increases renin expression in the

kidney, heart, and adrenal but not in the submandibular or genital glands. Androgens and estrogens increase extrarenal tissue renin mRNA levels but not renal mRNA. Renal renin expression has also been shown to be regulated by alterations in the level of angiotensin II via a negative feedback mechanism. Treatment of transgenic mice bearing the human renin gene with the converting enzyme inhibitor captopril resulted in a 5-10 fold increase in renin expression in the kidney[21]. Similarly, enalapril treatment of rats increased renal renin mRNA expression, which could be reversed by infusion of angiotensin II[22]. Further regulation of renin levels occurs by control of the synthesis and secretion of prorenin, such as by cAMP, and/or the conversion of prorenin to renin[10].

Angiotensin-Converting-Enzyme (ACE)

Angiotensin-Converting-Enzyme (ACE) is an endothelium-bound, dipeptidyl carboxypeptidase that converts angiotensin I to the potent vasoconstrictor angiotensin II and also inactivates the vasodilator bradykinin[10]. ACE has been found to be present in nearly all mammalian tissues and body fluids. The highest levels of ACE activity in humans have been found in the lung, kidney, ileum, duodenum and uterus, with lower levels in the prostate, jejunum, testis, and adrenals[23]. Little is known about the regulation of ACE mRNA expression. ACE can be induced by corticosteroids in cultured endothelial cells and alveolar macrophages[23].

Hyperthyroidism tends to elevate levels of circulating ACE.

Angiotensin II and Angiotensin II Receptors

The octapeptide angiotensin II is the main effector of the RAS. Certain peptide fragments, particularly Angiotensin III(2-8) and possibly Ang(1-7) and Ang(3-6), also have biological activity[24]. Angiotensin II, itself, has profound effects on cardiovascular function. Its multiple biological actions in various target tissues include the following effects: induction of vasoconstriction and a subsequent increase in peripheral vascular resistance and arterial blood pressure; stimulation of aldosterone release from the zona glomerulosa cells of the adrenal cortex; stimulation of sodium and fluid reabsorption from the proximal tubules of the kidney; induction of thirst and sodium appetite; facilitation of norepinephrine release from the noradrenergic nerve endings; and stimulation of cellular growth in vascular and nonvascular smooth muscle and renal proximal tubular epithelium (Figure 1-2)[1]. Blocking Ang II production will reverse these effects, leading to renal vasodilation, and an increase in glomerular filtration rate and sodium excretion.

The effects of angiotensin II are modulated by an interaction with its receptors, which are present in different tissues. Two subtypes of angiotensin II receptors are identified based on their differential affinities for nonpeptide drugs[26]. The angiotensin II type I (AT1)

receptors have a high affinity for losartan, whereas AT2 receptors have a high affinity for PD123177. The AT1 subtype is the classical angiotensin II receptor which mediates the well known effects of this peptide[25]. The AT1 receptor mRNA has been detected in vascular smooth muscle cells, kidney, adrenal, liver, heart, aorta, lung, spleen, uterus, ovary, and specific regions of the brain. The AT2 receptor is expressed in the rat adrenal medulla, the inferior olive of the brain and in the fetus[26]. The expression of angiotensin II receptors is influenced by circulating angiotensin II levels, dietary sodium intake and hormonal factors. Angiotensin II has been shown to reduce AT1 receptor mRNA expression by 50% after 4-6 hours in cultured vascular smooth muscle cells[10]. Low sodium intake down-regulates angiotensin II receptors while high sodium intake up-regulates the receptors[10]. Glucocorticoids, estrogen, and insulin have also been shown to modulate Ang II receptors[10].

The AT1 receptor is a member of the superfamily of G protein-coupled receptors that have seven transmembrane regions[26]. The signal transduction pathway is initiated by the binding of angiotensin II with its receptor, which then activates phospholipase C-mediated breakdown of the membrane phosphatidylinositol biphosphate (PIP) to generate inositol triphosphate(IP3) and diacylglycerol(DG). IP3 is released into the cytosol and brings about the mobilization of intra-

cellular calcium. DG is retained in the cell membrane where it activates a protein kinase C that is linked to an

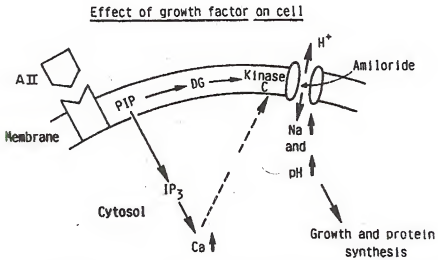


Figure 1-3. Signal transduction of agiotensin II receptor type I. PIP:phosphatidylinositol biphosphate; IP_3: Inositol triphosphate; DG:diacylglycerol(adapted from reference 1).

amiloride-sensitive Na^+-H^+ exchanger, resulting in an increase in intracellular pH and the promotion of growth and protein synthesis (Figure 1-3)[1].

The function of AT2 receptors is less well known than that of AT1. AT2 receptors are more abundant in embryonic and neonatal tissues than in adults, suggesting a role in development. The signaling transduction pathway of the AT2 receptor is much less elucidated and it does not appear to be G-protein-linked[26].

Angiotensinogen

Angiotensinogen(AGT), a 452 amino acid glycoprotein, is the precursor of the RAS, and the major substrate of renin. Angiotensin I is produced by renin cleavage of a leucine-

valine bond of the N-terminal region in the human angiotensinogen or a leucine-leucine bond in the angiotensinogens of other species[27,28]. The majority of circulating angiotensinogen is most likely derived from the liver, in particular the pericentral zone of the liver lobules. Adipocytes and astrocytes also produce small amounts of AGT. Unlike renin, whose secretion is highly regulated, newly synthesized AGT is released into the blood stream in a constitutive manner[28,29]. The plasma serves as the major reservoir of this protein, and plasma and cerebrospinal fluid concentrations are approximately 1 μM and 0.2 μM, respectively[27]. At the molecular level, glucocorticoid, estrogen and thyroid hormones increase angiotensinogen mRNA in rat hepatocytes[30-33]. Transcriptional activity of the AGT gene is highly dependent on the upstream (5' end of the transcriptional initiation site) cis-acting DNA which responds to these hormones and cytokines (Figure 1-4)[10]. This region also contains an enhancer element and confers tissue specificity on AGT gene expression. AGT mRNA has been found to be abundant in liver, fat, and brain cells and has been detected in small amounts in the lung, kidney, ovary, adrenal gland, heart, spinal cord, and testes. Individual AGT secreting tissues may have higher levels of AGT than those that receive this protein only from circulation[34,35].

The Role of AGT in Hypertension

Several observations point to the relationship between AGT and blood pressure[36-42]. Clinical studies have found statistically significant correlations between plasma Concentrations of AGT and blood pressure in human subjects ($r=0.39$, $p<10^{-6}$); higher plasma concentrations of AGT have

ANGIOTENSINOGEN (Human)

Figure 1-4. Schematic representation of 5' flanking regions of the AGT gene. GRE: glucocorticoid response element; ERE: estrogen response element; TRE: thyroid hormone response element; APRE: acute-phase response element; ENH: enhancer region; PAL: palindromic sequence(adapted from reference 10)

been found in hypertensive subjects and in the offspring of hypertensive parents, compared with normotensives[37]. A genetic linkage between molecular variants of AGT such as T174M, M235T and essential hypertension were also observed [39,40].

The SHR model of essential hypertension are found to have a higher plasma AGT levels than normotensive Wistar Kyoto rats at 14 weeks of age[42]. Blood pressure also can be decreased after administration of AGT antibodies and increased in transgenic animals overexpressing AGT[38].

Despite this evidence, the role of AGT in hypertension is still debatable. Most arguments are based on the extracellular nature of AGT that makes it a major reservoir for

the action of renin. It is generally considered that small changes in AGT concentration would not affect the concentration of functioning angiotensin II, thus an exclusively AGT-dependent hypertension is theoretically difficult to imagine[28]. However, based on enzyme kinetic studies, plasma AGT concentrations in the rat and human are about 1 μ mol/L. To reach a zero-order enzymatic reaction, ten times more AGT than naturally present is required[36]. This suggests that the large amount of AGT present in plasma does not provide an excess of substrate for renin and a rise or fall in renin substrate can lead to a parallel change in the formation of angiotensin II.

Feedback Interactions Involved in RAS

The feedback interactions of different components of the RAS have been investigated[43-55]. By using experimental strategies such as transgenic animals, antisense technology, or by surgical ablation of organs involved in blood pressure regulation, it has been possible to dissect the multiple components that underlie primary hypertension[56-58].

By using gene targeting via homologous recombination, one can abolish or knock out a defined genetic locus or mutate a particular set of nucleotides that encodes a peptide domain of interest[57]. This technique has been used to define the exact role of genes that underlie normal cardiovascular function. Tanimoto, et al.[43], generated angiotensinogen-deficient mice by homologous recombination in mouse

embryonic stem cells. These mice do not produce hepatic angiotensinogen, resulting in a complete loss of plasma immunoactive angiotensin I. The systolic blood pressure of the homozygous mutant mice was 66.9±4.1 mmHg, which was significantly lower than that of the wild-type mice (100.4± 4.4 mmHg). This profound hypotension in angiotensinogen-deficient rats demonstrates an indispensable role for the RAS in maintaining blood pressure. In contrast, Kimura, et al.[44], generated transgenic mice by injecting the entire rat angiotensinogen gene into the germline of mice. The transgenic line developed hypertension and both total plasma angiotensinogen and angiotensin II concentration were three fold higher than in the control. In situ hybridization showed higher mRNA in the liver and the brain of these transgenic animals.

Fukamizu, et al.[45], constructed the chimeric renin-angiotensin cascade in mice comprising both human renin and angiotensinogen as well as the endogenous angiotensin converting enzyme and angiotensin II receptor by cross-mating separate lines of transgenic mice carrying either the human angiotensinogen or human renin gene. Neither single gene carrier developed hypertension despite the observed normal tissue-specific expression of the transgenes. Dual gene strains exhibited a chronically sustained increase in blood pressure. Administration of a human renin-specific inhibitor (ES-8891) effectively reduced the elevated blood pressure only in the cross-mated hybrid mice, but treatment

with the angiotensin converting enzyme inhibitor captopril and a selective antagonist (DuP753), directed at the angiotensin II receptor, decreased the basal level of blood pressure in the single gene carriers as well as in the dual gene mice[45]. These results demonstrated that the sustained increase in blood pressure of the hybrid was initiated by an interaction between the products of the two human genes.

Schunkert, et al.[47], investigated the feedback regulation of RAS by studying the effect of angiotensin II on the regulation of ACE gene expression and enzymatic activity. Angiotensin II infusions increased plasma Ang II concentration and mean arterial blood pressure and decreased ACE mRNA levels in the lung and testis, two major sites of ACE synthesis. There was less pronounced but parallel decreases in pulmonary ACE activity while serum and testicular ACE activity displayed only minimal changes. This data would suggest that pulmonary ACE expression is subject to negative feedback by angiotensin II. Angio-tensin II infusion suppressed plasma renin concentration, kidney renin concentration, and renal renin mRNA levels in a dose dependent manner. In contrast, angiotensin II infusion increased renal AGT mRNA and also increased liver AGT mRNA levels and plasma AGT concentration. These data suggest that plasma angiotensin II up-regulates renal AGT and down-regulates renal renin gene expression, a reciprocal feedback regulation which may have important physiological consequences.

Moreover, Dzau, et al.[48], found that infusion of angiotensin II increases the AGT release rate while infusion of angiotensin I had no effect. Direct infusion of renin in rats treated with captopril resulted in a further suppression of the AGT release rate, suggesting that renin inhibits AGT release whereas angiotensin II stimulates it.

The interaction of the RAS also was studied by means of nephrectomy combined with adrenalectomy. Hilgenfeldt, et al. [49], studied the changes in AGT, angiotensin I and plasma renin concentration after ablation of kidney and adrenals. Plasma AGT levels were shown to increase approximately 5-fold after 24 hours in nephrectomized rats, and pretreatment with β-adrenoceptor atenolol blunted this increase. The angiotensin II receptor antagonist Dup-753 also abolished the increase in AGT and nephrectomy plus adrenalectomy also blunted the rise in plasma AGT. Nephrectomy alone induced a 5-fold increase of AGT mRNA in liver and a 2.6-fold increase was observed with nephrectomy and adrenalectomy. These results suggest that the increase in plasma AGT after nephrectomy be essentially mediated by angiotensin II via an unknown adrenal mechanism.

Current Antihypertensive Drug Therapies

Antihypertensive drugs exert their effects by interfering with normal blood pressure regulatory mechanisms. Pharmaceutical agents are categorized based on the principle regulatory site or mechanisms by which they act[59,60].

There are several antihypertensive agents such as diuretics, sympathoplegic agents, and vasodilators as well as types of RAS inhibiotrs. Diuretics lower blood pressure by depleting the body's sodium and reducing blood volume. Sympathoplegic agents lower blood pressure by reducing peripheral vascular resistance, inhibiting cardiac function, and increasing venous pooling in capacitance vessels. Direct vasodilators reduce pressure by relaxing vascular smooth muscle thus dilating resistance vessels and increasing capacitance. Calcium antagonists lower blood pressure by interfering with calcium-dependent contractions of vascular smooth muscle, thereby decreasing peripheral vascular resistance[59].

Four types of drugs which interrupt the RAS at different sites have been proven to be effective in decreasing blood pressure in hypertensive patients (Figure 1-5)[1]. β-Adrenergic blockers lower blood pressure by decreasing cardiac output associated with bradycardia and depresses the RAS by inhibiting the stimulation of renin production by catecholamines[59]; Renin inhibitors inhibit the release of renin; ACE inhibitors inhibit the enzyme that hydrolyzes angiotensin I to angiotensin II[59]; The angiotensin II receptor antagonists competitively block the effect of angiotensin II at its receptor sites[59]. The current anti-hypertensive drug therapies are not optimal. Problems such as unpleasant side effects, short half lives of the molecules and poor patient compliance result in noneffective therapy despite the potential pharmacological effectiveness

of each of these agents. Hence, the development of a long term, more specific therapeutic agent would greatly benefit patients and ultimately reduce health care costs.

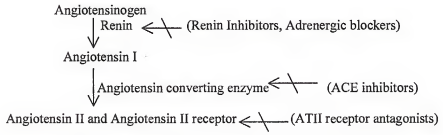


Figure 1-5. Antihypertensive drugs working on the RAS (adapted from reference 1).

The Spontaneously Hypertensive Rat as an Animal Model of Essential Hypertension

The spontaneously hypertensive rat(SHR) has been used to study the mechanisms of essential hypertension. This model was first introduced in 1963 by Okamoto and Aoki[61]. The colony was started by mating a male Wistar-Kyoto (WKY) rat with elevated blood pressure (145-175 mmHg) with a female WKY rat with slightly higher than average blood pressure (130-140 mmHg). They then conducted brother-sister inbreeding of siblings, selected for having the highest pressures in each litter. After the third generation, these rats, without exception, spontaneously developed hypertension as early as several months after birth.

By 1969, the group had successfully developed an inbred strain of SHR in which homozygosity had been achieved in more than 99% of all genetic loci. The absence of genetic variation among the individuals of the inbred strain made it

a powerful tool to study the determinants of elevated blood pressure. Similar to most patients with essential hypertension, SHR have normal or lower plasma renin activity and plasma angiotensin II concentrations relative to their normotensive counterparts-the WKY model[41]. Administration of ACE inhibitors or anti-renin antibodies decreased blood pressure in SHR. These results suggest a possible role of RAS in the maintenance of their hypertensive blood pressure.

A recent study suggested that an abnormality in the regulation of AGT gene expression might be involved in the development of hypertension in SHR[61]. It has been shown that, even though plasma AGT concentration in the SHR was comparable to that of WKY at six weeks of age, the level increased significantly at 14 weeks of age and was higher than the WKY. Brain AGT expression in SHR was higher than WKY at 6 weeks of age and was comparable to that of WKY at 14 weeks of age. Cardiac and fat AGT mRNA levels also were significantly higher at 14 weeks of age in SHR than in WKY [42]. An alteration of AGT expression by sodium also has been observed in the SHR[62].

Gene Targeting in Hypertension Research and Treatment

Gene targeting techniques have been used both as research tools in hypertension research as well as therapeutic agents for other disease states. Disruption of the expression of genes involved in hypertension such as angiotensinogen [63, 64,66,68] and the angiotensin II receptor[65] have been

successful in decreasing blood pressure. Kallikrein gene therapy has been shown to decrease blood pressure in a hypertensive rat model[67]. Recently, Wielbo, et al. showed that central administration of ASODN targeted to AGT mRNA significantly decreased blood pressure in the SHR for a prolonged period of time (6 days) with corresponding decreases in hypothalamic angiotensin II and AGT levels[64]. Blood pressure has also been shown to decrease after interruption of peripheral RAS gene expression. Tomita et al.[63], were able to decrease blood pressure by using liposomes with a viral fusion protein mediated gene transfection technique. A transient decrease in plasma AGT and a concurrent decrease in blood pressure and plasma angiotensin II concentration were observed after giving the antisense RNA via the portal vein. Studies conducted by Wielbo, et al., showed significant decreases in blood pressure following the intra-arterial administration of a liposome-encapsulated antisense molecule targeted to AGT mRNA. The plasma Ang II and AGT levels also were decreased[68].

Antisense Oligodeoxynucleotides

Antisense oligodeoxynucleotides (ASODN) are short, single stranded sequences of DNA molecules that, by forming specific hydrogen bonds with complementary mRNA or DNA molecules, allow the specific regulation of gene expression [69]. Based on the simple Watson-Crick base-pairing rule, one can design ASODNs to target any gene with a known

sequence. A major advantage of this strategy is the specificity of the ASODN action. Theoretically, an ODN of 15-17 nucleotides in length should interact with only one target gene in the entire human genome[70]. In principle, an oligonucleotide(ODN) can be designed to target any single gene within the entire human genome, with the potential to create specific therapy for any disease in which the causative gene is known[71-73]. It also has the advantage of being used as a research tool to investigate the role of a particular gene in a physiological or pharmacological system.

ASODN activity was demonstrated in numerous biological systems[70,71]. Viruses represent the most attractive therapeutic targets since their genetic sequences are unique with respect to the human host. ASODN have shown activity against HIV, HSV 1 and 2, HPV and influenza in vitro[71]. They also have been reported to inhibit a variety of oncogenes including c-RAS and c-myc. Inhibition of gene expression is generally seen at high concentrations of ODNs[72].

Theoretically, oligonucleotides can be designed to target and interfere with every stage of gene expression. For example, they can be designed to target and bind to double-stranded DNA resulting in a triple helix formation with the subsequent inhibition of transcription, or by hybridization to nascent RNA. They can be designed to interfere with RNA splicing and transport of mRNA from the nucleus to cytoplasm through hybridization at intron-exon junctions. Translation can be inhibited by targeting the antisense to the AUG

initiation codon, thereby inhibiting the assembly of ribosomal subunits and the subsequent reading of the messages to be translated (Figure 1-6)[73].

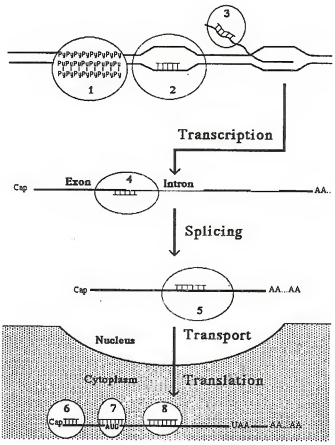


Figure 1-6. Summary of the possible sites of sequence-specific actions of ASODN. ASODN could interfere with transcription by (1) hybridization to the locally opened loop created by RNA polymerase; (2) hybridization to nascent RNA; interfere with splicing through (3) hybridization at intron-exon junctions; (4) interfere with transport of mRNA from nucleus to cytoplasm (5) interfere with translation through inhibition of binding of initiation factors; (7) inhibition of the assembly of ribosomal subunits at the start codon (8) or inhibition of ribosome sliding along the coding sequence of the mRNA (excerpted from reference 73).

Despite the observed activities, the mechanism of ASODN action is still unclear. It has been proposed that ASODN

inhibit gene expression through two distinct mechanisms[74]. One mechanism suggests that once the antisense molecules enter the cells, they bind to its target mRNA in either the cytoplasm, nucleus or both. Following this hybridization, cellular RNase H protects the cells by cleaving the RNA portion of the RNA:DNA duplex. Once cleaved, the mRNA is no longer competent for translation and may be rapidly degraded. This mechanism has an advantage in that each message is permanently inactivated upon cleavage and each ODN can inhibit multiple copies of each target mRNA. This cleavage, however, may have the disadvantage of being nonspecific, since the transient hybridization to other mRNAs may activate RNase H as well[75]. In the second antisense mechanism, the binding of an ODN to a target mRNA inhibits gene expression through simple steric blocking. The ODN:RNA duplex forms and physically prevents the RNA from interacting with cellular components such as ribosomes, thereby inhibiting translation of the RNA into its specific protein[74].

Thermodynamics of DNA:RNA Duplexes Formation

The relative stability of a nucleic acid duplex is measured by the melting temperature; the higher the melting temperature, the more stable the duplex[76]. Factors such as the length of the ODN, its AT/CG composition, and the base sequence all contribute to its stability[74]. On average, duplex stability is proportional to the number of base

pairs; the longer the duplex, the higher the stability. However, as the length increases, the affinity for closely related sequences also increases and the specificity may begin to decrease. Theoretically, the length of an ODN that should satisfy both stability and specificity requirement is as short as ten to fifteen bases. The stability of the duplex also increases as the G,C content of the ODN increases, due to their stronger hydrogen binding[76]. The development of ASODN as therapeutic agents has not been as smooth as once anticipated. Several requirements have to be met to ensure its practical use, such as large scale synthesis, in vitro and in vivo stability, successful delivery and specificity in its action[77,78].

Stability

The ODNs initially used in physiological studies were naturally occurring phosphodiesters. The linkages in these molecules are susceptible to degradation by endogenous serum and intracellular nucleases. An in vitro assay demonstrated that after microinjection into Xenopus embryos, phosphodiester ODNs have an intracellular half-life of less than 30 minutes[77]. Protection from degradation was achieved by use of a 3'-end cap strategy in which nuclease-resistant linkages were substituted for phosphodiester linkages at the 3' end of the ODN. Phosphorothioate analogs have a sulfur substituted for one or both nonbridging oxygens(Figure 1-7)[74]. This modification makes the

molecule more resistant to nuclease degradation, extending the half-life in vitro to 12 hours.

Permeation

Further problems encountered with the use of ODNs include poor cellular uptake and intracellular compartmentalization. Unlike many other small organic drug molecules of low molecular weight, ODNs (15 to 28-mer) are polyanionic hydrophilic molecules with a molecular weight range of 5000-10,000 and cannot passively diffuse across Cell membranes. Although the mechanism by which the ODNs enter the cells is not clearly identified, it is generally

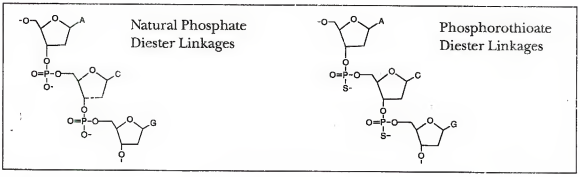


Figure 1-7. The structures of phosphodiester and phosphorothioate molecules (adapted from reference 74).

believed that the molecules enter the cells by a pinocytotic or receptor-mediated endocytotic mechanism[79-82]. In this process, molecules to be internalized first bind to specific receptors on the cell surface. These receptor/ligand complexes then become clustered in specialized areas of the plasma membrane, termed coated pits, and then become

invaginated to form a coated vesicle. The coat is rapidly removed. Fusion with the early endosome exposes the receptor /ligand complexes to lower pH which causes dissociation of ligand and receptor. Ligands may be transferred through late endosomes to lysosomes while the receptors which recycle are returned to the cell surface[83].

The cellular uptake of ODNs was studied in the HL60 cell line. Phosphorothioate ODN was demonstrated to be internalized by the process of adsorptive endocytosis and fluid-phase pinocytosis. The process is slowed by the metabolic inhibitor of the cells and is temperature-dependent[84].

The intracellular distribution of the ODN has been studied using fluorescence microscopy. A microinjection method was used to avoid the problem of the internalization pathway and fluorescently-labeled ODNs were observed to accumulate in the nucleus soon after injection. When fluorescently labeled ODNs are placed in tissue culture media, the fluorescence accumulates in vacuoles within the cell, forming a punctate perinuclear pattern which are presumably endosomal and lysosomal in nature. Weak visible fluorescence in the nucleus has been observed, suggesting that the release of ODNs from vacuoles is an inefficient process[82]. This observation supported the view that the major limiting factor in effective ODN delivery appears to be the escape of the ODNs from the endosomes and lysosomes where they are rapidly degraded by hydrolytic enzymes. The uptake of fluorescently labeled ODNs was enhanced by

coadministration with cationic lipids, demonstrated by the presence of fluorescence in the nucleus and concurrent increase in ODN activity[74].

Chemical Modification of the ASODNs

Efforts have been made to improve the properties of ODNs by chemical modification. Despite the fundamental Watson-Crick hydrogen-bonding scheme, which is central to the formation of the double helix and is unlikely to change substantially, all other structural features of the phosphodiester backbone, heterocyclic bases and sugars have been modified or replaced [74,85].

Modification of the phosphodiester backbone has been employed to improve stability, allowing for enhanced affinity and increased cellular permeation of ODNs. The properties of some of the phosphodiester backbone analogues are listed in Table 1-1[74].

In addition to phosphorothioation, mentioned previously, other linkages have been studied. Methylphosphonate-modified ODNs, which substitute the non-bridged oxygen with a methyl group, have the advantages of being neutral and providing better cellular uptake. But both of these two modifications suffer from decreased affinity to target sequences which affect their activity[74]. The chiral properties of ODNs containing either of these modifications was suggested to be a factor that affects its activity. Each of these isomers consists of a mixture of 2^n diastereomers (where n is the

number of linkages) and it is reported that pure R or pure S isomers would hybridize with different affinity[74]. Lesnikowski, et al. showed that for a 7-mer oligothymidine with a methylphosphonate backbone, the all-RP (R diastereomer of phosphorus) ODN had a significantly higher melting temperature than ODNs which are a mixture of diastereomers [86].

Sugar modification also has been used to enhance stability and affinity. Modification of the 2'-OH of the ribose sugar to form 2'-O-methyl or 2'-O-allyl sugar within oligonucleotides is found to enhance resistance to degradation without compromising affinity[74]. Heterocyclic

Table 1-1. Properties of selected phosphodiester backbone analogues (adapted from reference 74).

backbone analogue	activation of RNase H	resistance to nuclease	Chiral center
	Phosphorus Analogues		
phosphodiester	Yes	--	No
phosphorothioate	Yes	+	Yes
phosphorodithioate	Yes	++	No
methylphosphonate	No	++	Yes
phosphoramidate	No	+	Yes
alkyl phosphotriester	No	+	Yes
	Non-Phosphorus Analogues		
sulfamate	No	++	No
3'-thioformacetal	No	++	No
methylene(methylimino)(MMI)	No	++	No
3'-N-carbamate	No	++	No
morpholino carbamate	No	++	No
peptide nucleic acids(PNAs)	No	++	No

base modifications offer an opportunity to enhance the affinity without compromising RNase H cleavage of the target

RNA. 2-Amino-2'-deoxyadenosine introduces a third hydrogen bond into an A:T base pair which stabilizes duplex formation[74].

Modifications to enhance permeation by conjugation of an ODN to transferrin, a protein ligand for a cellular receptor, was shown to dramatically increase cellular association of ODNs[87]. Increased cellular association and activity have also been reported for an ODN-asialoglycoprotein conjugate targeted to the hepatitis B virus[88]. Fluorescein labeled ODN bound to streptavidin, which had 12 mannose residues attached, was found to be internalized preferentially in liver cells via cellular mannose receptors. Poly(L-lysine), a polycationic drug carrier, was also shown to increase both the rate and extent of cellular uptake of ODNs[89]. The attachment of hydrophobic molecules, such as cholesterol and phospholipids to the ODNs also has been reported to increase cellular uptake[74].

Liposomes as a Delivery Vehicle for ASODN

The use of liposomes as a carrier system for nucleic acid has received increasing attention[90-93]. Liposomes are vesicles in which an aqueous volume is enclosed by a membrane composed of lipid molecules with hydrophilic polar heads and hydrophobic nonpolar tails[91]. The membranes exhibit a variety of surface properties such as charge, membrane rigidity, and phase behavior[93]. In small organic molecule delivery only the self-closed bilayer vesicles, the

liposome is considered. In gene transfer several other structures and phases, such as the open lipid bilayer fragment, the inverse hexagonal phase and the micelle, are also important (Figure 1-8) [94].

Liposomes in Biological Systems

Liposomes administered in vivo are subjected to physiological interactions that determine the rate of clearance and degree of organ uptake. The major limitation of liposomes for pharmaceutical applications is their unpre-

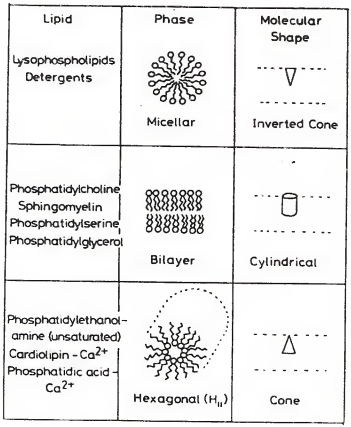


Figure 1-8. Phase structures formed by lipids in the aqueous solutions (adapted from reference 101).

dictable behavior in the body, such as: rapid clearance from the blood, restricted control of the encapsulated molecule release, low or nonreproducible drug loading and physical or chemical instability[95].

Circulating liposomes are taken up to a large extent by organs rich in the cells of the reticulo-endothelial system (RES), such as the liver, spleen, lung, lymph nodes and bone marrow. Larger liposomes of conventional formulation are rapidly removed from the circulation following intravenous injection by uptake primarily into Kupffer cells of the liver and macrophages in the spleen and lung. This passive targeting to phagocytic cells has been used for treating diseases of the RES such as liver leishmaniasis and fungal infections[95]. Small liposomes with diameters less than 0.1 μm can pass through fenestrated endothelium and gain access to liver parenchymal cells[96,97].

For therapeutic applications involving non-RES organs, prolonged blood circulation has been achieved by mimicking the composition of the red blood cell membrane. Inclusion of phospholipids with a synthetic hydrophilic polymer headgroup, such as a polyethyleneglycol chain, reduce the recognition of liposomes by the mononuclear phagocytic system and hence increases its circulation time[98]. Specific organ targeting also can be achieved by incorporation of certain ligands. Liposome containing lactosylceramide was shown to increase the transfection efficiency by a factor of 1000 in HepG2 cells through

interaction with asialoglycoprotein receptors on the cell membranes[99].

The physical integrity of liposomes can be modulated by changing the lipid composition. Increase in cholesterol composition has been shown to stabilize the bilayer and decrease the permeability of phosphatidylcholine liposome [100].

Methods for DNA encapsulation include reverse-phase evaporation(REV), sonication, Ca2+-EDTA chelation, cationic lipid complexes, detergent dialysis and viral envelope reconstitution[101]. Increased DNA encapsulation efficiency has been achieved by altering the physical state of the DNA such as condensing the DNA with bacteriophage protein or small organic molecules. In the REV method, multiple freeze-thawing and rehydration cycles, during which bilayers open and close, can make more molecules permeate into the interior of the liposome thus improving the encapsulation efficiency[101].

The Interactions of Liposomes with Cells

The mechanism of liposome-cell interaction and the effect of liposome structure and composition on its association with the cell is not completely understood. Different modes of interaction have been summarized in the literature(Figure 1-9)[102,103]. The endocytosis/phagocytosis mechanism proposes that cells with phagocytic activity take up liposomes into endosomes, endosomes then fuse with lysosomes to form secondary lysosomes where degradation takes place in

low pH (4.5) environments. Liposome phospholipids are then hydrolyzed to fatty acids and recycled and reincorporated into the host phospholipid. The content of the aqueous compartment is released after the membrane disintegrates. They may either remain sequestered in the lysosome until exocytosis or they will slowly leak out of the lysosome and gain access to the rest of the cell. Liposomes may also be taken up by receptor-mediated endocytosis[108].

Liposomes coated with low-density lipoproteins or

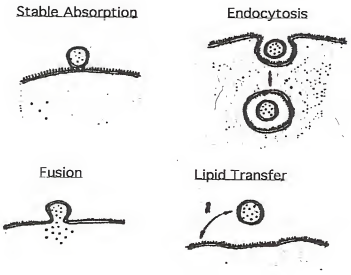


Figure 1-9. Possible mechanisms of interaction between liposome and cell surface (adapted from reference 102).

transferrin bind to the cell via surface receptors for these moieties and then are internalized via coated pits with subsequent ligand degradation, or recycling[104]. Inter-membrane transfer of lipid components can take place upon the close approach of the two phospholipid bilayers without disruption of the membrane's integrity[102].

Contact-release of the aqueous contents of liposomes occurs by a poorly understood mechanism in which contact with the cell causes an increase in permeability of the liposome membrane. This mechanism provides the means for introducing materials into specific cells without the need for ingestion of the whole liposome, and would be of particular value for cells which are not actively phagocytic[102]. Adsorption may take place either as a result of physical attractive force or as a result of binding by specific receptors to ligands on the vesicle membrane. Fusion results in incorporation of the liposomal lipids into the plasma membrane of the cell and diffusion of the liposome-trapped contents into the cytoplasm[102]. Incorporation of fusogens such as lysolecithin, detergent, surfactant or Sendai virus fusion proteins into the membrane have been shown to facilitate the liposome-cell fusion process[105].

Liposomes have been used as a tool to deliver oligonucleotides. Liposome encapsulation can improve the passage of ODNs through the cell membrane as well as protect them in the extracellular medium. It is also possible to target liposomes to specific cell populations by coupling certain proteins or antibodies on their surface[107]. Liposome structure and surface properties determine several different ways liposomes can interact with ODNs. ODNs can be encap-sulated in the liposome interior, bound onto the liposome surface, or embeded between bilayers[106]. Liposomes have been differentiated based on the mode of

liposome inter-action with their target cells[107]. Conventional liposomes are formulated with phosphotidylcholine and cholesterol are examples of this. These are also referred to as non-targeting liposomes. Liposomes which are pH-sensitive were designed to release their content as they pass through regions of low pH. They can be used to take advantage of the pH gradient of the endocytic process, to avoid lysosomal degradation and to improve the intracellular delivery of macromolecules[109-112]. One way to make pH sensitive liposomes is to reconstitute it with a viruse such as vesicular stomatitis virus or with influenza virus membrane glycoproteins. These proteins undergo conformational changes at low pH levels and promote acid-induced liposome-cell fusion and increased delivery of the encapsulated contents into the cytoplasm[105]. The poly-morphic phase behavior of some unsaturated lipids provides another way to make pH-sensitive liposomes. The most commonly used lipid com-position of pH-sensitive liposome is dioleylphosphatidyl-ethanolamine(DOPE) which has ionizable headgroups. At low pH, the headgroup becomes protonated and forms the inverted hexagonal (HII) phase rather than the bilayers observed under normal physiological pH[112]. The inverted molecules fuse more readily with the endosomal membrane and leads to the release of the liposome contents. Immunoliposomes refer to liposomes linked to antibodies that are designed to target cells which express sufficient and specific antigens [113]. This method

has been applied to treat tumor cells by incorporating in the liposome the antibodies against folate protein, which has over a 20-fold higher expression in tumor than in normal cells and was shown to give more specific targeting to tumor cells.

Cationic liposomes as a DNA carrier system was first reported by Felger, et al. using the synthetic cationic lipid N-[1-(2,3-dioley]oxy)propyl]-N,N,N-rimethylammoniumn chlo-ride (DOTMA) in combination with DOPE[114]. The positively charged cationic liposomes form a complex with the negatively charged DNA. These complexes contain excess cationic lipids which neutralize the negative charge of the DNA and provide the complex with a net positive charge allowing interaction with negatively charged cell surface. Substitution with negatively charged lipids was shown to suppress the delivery[115,116].

Hypothesis

Based on these information, our hypothesis is: inhibition of angiotensinogen by antisense oligodeoxynucleotide to angiotensinogen mRNA attenuates hypertension in the SHR rat model, and this blood pressure decrease may be prolonged via optimization of route of ASODN administration and optimization of oligonucleotide delivery. To test this hypothesis, both *in vitro* and *in vivo* studies will be conducted.

CHAPTER 2
GENERAL MATERIAL AND METHODS

Animals

Male, spontaneously hypertensive rats(SHR), Sprague Dawley and Wistar-Kyoto(WKY) rats weighing between 250-275g (Harlan, Indianapolis, Ind.), were kept in the University of Florida Animal Care Facilities. They were housed in a room with a 12-hour light-dark cycle and fed on standard laboratory rat chow and tap water ad libitum. Rats were accommodated for one week before experiments began.

Arterial and Venous Cannulation

Animals were anesthetized with ketamine/xylazine (100 mg ketamine + 20 mg xylazine/ml at a dose of 0.5-0.7 ml/kg, IP) and a heparinized (100U/ml) catheter made of PE50 tubing (0.58 mmID, 0.965 mmOD) was inserted into the left carotid artery, 25 mm toward the heart. In addition, a catheter was inserted into the femoral vein and extended 60 mm into the dorsal vena cava. The catheter dead space was filled with heparin (1000U/ml) to maintain patency. Both catheters were tunneled under the skin and exteriorized between the scapulae and plugged with stoppers. Animals were allowed to

recover for 24 hours after catheterization before experimentation.

Blood Pressure Measurements

Blood pressure was measured by direct method or indirectly, using the tail cuff method. For the direct method, a catheter was inserted into the external carotid artery and connected to a pressure transducer that was interfaced with a Digi-Med BP Analyzer(micro-Med, Indianapolis, Ind.), Signals were recorded on a Gould TA2405 EasyGraf Physiograph, which provides information on systolic, diastolic and mean arterial pressure and heart rate. The indirect method refers to the tail-cuff plethysmography method[117,118]. The rats were first warmed for 15 minutes at 37°C in a thermostatically controlled heating cabinet for better detection of tail artery pulse. Then the rats were put in a holder with heating pad. Their tails were passed through an inflatable cuff and a rubber bulb connected to a pulse transducer was taped on the tail distal to the cuff. The pressure in the cuff was increased rapidly when inflated, until the tail pulse disappeared and then released slowly. When pressure in the tail arteries exceed that in the cuff, the pulse reappeared and systolic pressure was indicated by the level of the first pulse wave. Daily values were obtained by averaging 5-10 successive readings.

Cell Culture

H-4-II E, Hepatoma, Reuber H35, rat cells were purchased from ATCC (Rockville, Maryland). Cells were grown in a monolayer culture on 10 cm petri dishes in 12 ml of Eagles Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 10% calf serum and incubated in 95% air-5% CO2 at 37°C. The culture medium was changed every other day. Cells were passaged until confluent at a ratio of 1:6 using 1.0 ml of 0.25% trypsin-EDTA.

Oligodeoxynucleotides

The antisense oligodeoxynucleotide (ASODN) was designed based on the angiotensinogen(AGT) mRNA sequence published by Okhubo[119]. The ASODN is an 18-mer, complementary to the -5 to +13 base sequence of AGT mRNA, and covers the AUG translation start codon. The sequence is: 5'-CCGTGGGAGTCA TCACGG-3'. The scrambled ODN (ScrODN) has the same base composition but in random order: 5'-TCGCTAAGCGGCAGCGTG-3'. Both nucleotides were synthesized in the phosphorothioated form in the DNA Synthesis Laboratory, University of Florida.

Liposome Synthesis

Phosphatidylcholine-cholesterol liposomes were composed of 80% phosphatidylcholine and 20% cholesterol (Avanti Polar Lipids Inc., Alabaster Alabama). Liposomes were prepared by the reverse phase evaporation method[120]. The lipids were dried and dispersed by rotary evaporation and then rehydrated in phosphate buffer to form multilamellar

vesicles by mechanical shaking. The vesicles were subjected to ten freeze-thaw cycles to enhance ODN entrapment. Liposomes were then passed through a 0.1 μm filter membrane for size reduction.

Cationic liposomes were composed of dimethyl-dioctadecyl-ammonium bromide (DDAB) and dioleoylphosphatidyl-ethanol-amine(DOPE) (2:5,w/w) (Avanti Polar lipids, Alabaster Alabama). Lipids were dispersed and rehydrated in 1 ml deionized water and sonicated to reduce the size to about 100 nm. ASODNs were complexed with cationic liposomes by mixing at a -/+ charge ratio of ASODN/cationic lipids of 0.18 and incubated at room temperature for 30 minutes before experiments[121].

Northern Blot Analysis

mRNA was isolated using acidic guanidium thiocyanate-phenol-chloroform[122] and quantified by densitometry[123]. Cells or tissues were lysed using mercaptoethanol then reated with guanidium thiocyanate followed by phenol and chloroform extraction. RNA was then precipitated by isopropanol at -20^{0}C, evaporated and resuspended in sterile water. The concentration of total RNA was measured by spectrophotometry at a wavelength of 260 nm. An aliquot of 20 μg total mRNA was separated by electro-phoresis on an agarose formaldehyde gel at 25V for 16 hours. RNAs were then immobilized on nitrocellulose membranes by capillary transfer. The RNA was then subjected to prehybridization for

4 hours at 56°C with 1 x Denhardts SSPE solution, 5 x SSPE, 0.1% SDS, 50% formamide, and 250 mg/ml denatured salmon sperm DNA. Hybridization was carried out under the same conditions with a ^{32}P labeled riboprobe to the specific mRNA sequence. After stringency washes, the membrane was exposed to X-ray film then developed. Membranes were reprobed with Cathepsin D mRNA to ensure equal loading.

Angiotensinogen Assay

Aliquots of 500 ul of culture medium or rat plasma were evaporated to dryness. The dried samples were assayed for AGT by the direct radioimmunoassay method of Sernia[124]. AGT sample content was measured from a standard curve of pure rat AGT diluted in the same cell culture medium or plasma. The assay sensitivity was 0.3 ng/tube, with an inter-assay and intra-assay variability of 14% and 9%, respectively.

Angiotensin II Assay

Plasma samples were extracted using methanol and trifluoroacetate on C18 reverse phase extraction cartridges(Varian, Windham, NH). Angiotensin II(Ang II) was measured by double-radioimmunoassay (RK-A22, Alpco, Windham, NH). The samples were first incubated for 16 hours with an anti-angiotensin II antibody. The ^{125}I-Ang II competes with Ang II present in the samples and standards for the same antibody binding sites. A solid-phase second antibody is

then added and antibody-bound fraction is precipitated and counted on a Beckman DP550 Gamma counter. The sensitivity of the assay is 0.7 pg/ml Ang II and there is minimal cross-reaction with other peptides.

Reverse-transcriptase Polymerase Chain Reaction(RT-PCR)

RT-PCR was used to quantify messenger RNA. Total mRNA was first isolated, then converted to cDNA. The cDNA was then amplified by thermocycling. Total mRNA was isolated using acidic guanidium thiocyanate-phenol-chloroform. Then 5 ug of total mRNA was mixed with 10 mM dNTP, 0.1 μM $MgCl_2$, 100 μM oligo d(T), 40 U/ul of RNase inhibitor, and 50 U/ul of reverse transcriptase(Promega, Madison, WI) in a 50 ul volume. The reaction was incubated at 37°C for 1 hour, then heated at 95°C for 5 minutes, followed by a 5 minute incubation at 4°C. Five microliters of RT product was then subjected to PCR reaction. The PCR reaction was performed in a total volume of 50 ul in the presence of 10 mM of dNTP, 100 pmol/ul of primers, 5 U/ul of TAQ Polymerase and 0.025 M of $MgCl_2$ (Promega, Madison, WI). The thermocycle was programmed as:

Step 1: 5 minutes at 95°C

Step 2: 30 cycles at 1 minute at 94°C; 2 minutes at 58°C; 3 minutes at 72°C

Step 3: 10 minutes at 72°C

AGT and control genes were amplified in the same reaction to eliminate the factors that would affect the amplification

efficiency. The sizes of the two amplified products were AGT 463bp, β-actin 350bp. Ten microliters of each PCR reaction mixture were separated with 1.8% agarose gel using the electrophoresis method and stained with ethidium bromide and photographed. The density of the bands were then measured by densitometer(Biorad, Boston, MA) and the ratio of density between the AGT and control were plotted. The primer sequences for AGT were: Sense, CAACACCTACGTTCACTTCC and Antisense, GAGTTCAAGGAGGATGCTGT. The primer sequences for control β-actin were: Sense, AACCGCGAGAAGATGACCCAGATCATGTTT and Antisense, AGCAGCCGTGGCCATCTCTTGCTCGAAGTC[125].

Fluorimetric Analysis

Cells or weighed tissue were prepared for fluorimetric analysis of fluorescein isothio-cyanate (FITC)-conjugated oligonucleotide by homogenization in 1 ml PBS followed by centrifugation at 14,000 rpm for 10 minutes. The supernatant was aspirated and fluorescence activity was determined on a Perkin Elmer Luminescence Spectrometer, excitation:487 nm and emission:525 nm. The percentage of oligonucleotide present in each sample was determined by dividing the fluorescence of the sample by the fluorescence of a standard containing only 1 ml PBS and 2.5 μg FITC-conjugated oligonucleotide.

Confocal Microscopy

Microscopical analysis of FITC conjugated ASODN uptake and distribution was carried out on a Nikon Optiphot-2-

Fluorescent laser scanning confocal microscope in the Center for Structural Biology, at the University of Florida. Cells were grown on plain glass microscope slides. After treated with ASODN, PS/ASODN or CA/ASODN for four hours, cells were fixed using 2% formaldehyde and then slides were mounted using Gel Mount (Biorad, Boston, MA) and micrographs were obtained at excitation and emission wavelengths of 472 and 525 nm, respectively.

MTT Test

MTT[3-(4,5-dimethylthiazole-2-yl)2,5-diphenyl-tetrazolium bromide] test, described by Mossmann[126], was used as *an in vitro* test for cellular toxicity. The cells were grown in a 96 well culture plate. After a 48-hour incubation with ASODNs, cationic lipids or CA/ASODN complexes, the medium was decanted. A 10 ul MTT stock solution (5 mg/ml in PBS) was added to 0.1 ml culture medium. After 4 hour incubation at 37°C, the MTT cleavage product, formazan, was solubilized by the addition of 0.1 ml 0.04 N HCl prepared in isopropanol. The optical density of the product was measured using a reference wavelength of 630 nm and a test wavelength of 560 nm by microplate reader (Biorad, Boston, MA).

Aldosterone Analysis

Plasma samples were collected and stored at -20°C. Aldosterone was measured by radioimmunoassay (DSL-8600 ACTIVE Aldosterone Radioimmunoassay Coated-Tube Kit,

Diagnostic Systems Laboratories, Inc. Texas) The procedure is based on the basic principle of immunoassay where there is competition between a radioactive and non-radioactive antigen for a fixed number of antibody binding sites. The bound antigen is separated from free antigen by decanting the antibody coated tubes. The sensitivity of the assay was 25 pg/ml. Cross-reactivity to closely related naturally occurring steroids was reported as negligible.

Sodium-potassium Analysis

Urine samples were centrifuged and the supernatant was saved for analysis on a NOVA 1+1 Automated Sodium/Potassium Analyzer (NOVA Biomedical, Massachusetts). The principle of the assay is based on the electrical potential of the solution measured against a reference electrode.

Statistical Analysis

Statistical analysis was performed by ANOVA for treatment effect, and the Duncan multiple range test was used for individual comparisons. Data for individual time points were analyzed using the Students independent T-test. Significance was at the 95% confidence limit.

CHAPTER 3
LIPOSOME-MEDIATED OLIGONUCLEOTIDE DELIVERY
IN HEPATOMA CELL CULTURE

Specific Aims

The hepatoma H4 cell culture experiments seek to evaluate two specific aims. The first is to develop an efficient delivery mechanism for antisense oligonucleotide targeted to angiotensinogen mRNA for cells in culture; the second is to determine the uptake efficiency, cellular distribution, cytotoxicity, and effects of the antisense oligonucleotides on AGT mRNA and protein expression in hepatoma H4 cell culture.

Introduction

Pharmaceutical agents targeted to block the renin angiotensin system are effective in treating hypertension [59]. Currently there are no drugs available to block the precursor of the RAS, angiotensinogen. In our previous studies, ASODN was designed to hybridize to the AUG start codon of angiotensinogen mRNA[64,68]. This ASODN successfully decreased blood pressure in SHR when administered both centrally and peripherally. This treatment, however, did not return elevated blood pressures to normal levels and the effect lasted only for a limited time. One hurdle to ASODN

use is its poor tissue or cellular uptake[77]. Liposome vehicles were utilized to improve ASODN delivery by increasing the interaction of the ASODN with tissues or cells to protect the ASODN from degradation by intracellular enzymes[101].

Cationic liposomes have been shown to be effective for delivering oligonucleotides in cell culture[128]. Even though novel synthetic cationic lipids have been reported to provide higher efficiency in tested cell lines, in general no single cationic lipid formulation appears to be uniformly superior to others[129,130]. Transfection conditions must be optimized individually for each cationic liposome formulation and cell line. The following parameters may be modulated: the DNA to lipid ratio of the complex; the total dose of DNA:lipid complex added; the density and dividing stage of the cultured cells; the medium in which the cells are cultured; the duration of exposure of the liposome:DNA complexes to the cells; and the time points when the cells are analyzed[131].

Most cationic liposomes formulated contain two lipid species, a cationic amphiphile and a neutral phospholipid which functions as fusogen, typically dioleoyl-phosphatidyl-ethanolamine(DOPE). Cationic vesicles formulated without DOPE have been shown to be 2 to 5-fold less active than the one with DOPE[132]. The effect of DOPE is attributed to its capacity for transition from the bilayer phase into the inverted hexagonal phase, which leads to increased membrane

fusion[132,101]. Replacing DOPE with other neutral phospholipids of the same acyl chain with a choline head group instead of the ethanolamine such as, dioleoylphosphatidyl-choline (DOPC), abolish most of the transfection activity of the liposomes[133].

Compared with conventional liposomes, cationic liposomes do not require an encapsulation step that limits the application of the carrier. Instead, negatively charged ASODNs are directly mixed with preformed liposomes and form complexes through electrostatic interaction with cationic lipids[134]. Despite the fact that the physicochemical properties of the cationic lipids and ASODN complexes are poorly characterized, it is generally agreed that the charge ratio of the ASODNs to the cationic lipid is a critical parameter. The charge ratio determines such factors as compactness of the complex; the masking of the negative charges of the ASODNs, and the steric interaction of the complex with the cell membrane[135]. Numerous studies have shown that transfection efficiency changes dramatically with different ratios of DNA to cationic lipid[136,137].

The mechanism of cellular delivery of the cationic liposome/ODN complex is via an endocytotic process, mainly mediated by the mononuclear phagocyte system, but also by non-phagocytic cells such as fibroblasts, kidney cells, lymphocytes and hepatocytes[138]. The non-receptor-mediated endocytosis appears to be strictly dependent on the size of the liposomes. Tightly compacted, condensed small-sized

cationic liposome/ASODN complexes are more favorable for uptake by endocytosis. The optimal size of the complexes is 50-100 nm. Vesicles larger than 400 nm are not favored for endocytosis. The particle size of the cationic liposome/ODN complex ranges from 75 nm to >3000 nm, and depends on several factors such as: (i)the cationic lipid species; (ii)the amount of neutral co-lipid like DOPE; (iii)the cationic lipid/DNA ratio; (iv)the concentration of lipid and DNA in the final formulation; and (v)the composition of the suspending vehicle[129].

Successful gene inhibition by ASODN has been reported in various biological systems[70,71]. The observed biological effects have mainly been observed at high concentrations of ASODN, when some non-specific effects were produced[78]. Our previous in vitro transcription/translation study showed that a high doses of ODN (3-30μmol/l), both ASODN and control scrambled ODN caused a decrease in AGT expression [68]. By using cationic liposomes it may be possible to decrease the dose of ASODN required to produce the same biological effect while decreasing the potential for non-specific effects caused by ASODN.

Cationic lipids such as DDAB have been reported to be highly toxic to cells. They are similar to surfactant molecules. At high concentrations, these lipids cause cell membrane disruption and poration[139]. Because of this, the toxicity of cationic lipids, ASODN alone and the ASODN-lipid complexes were determined by the [3-(4,5-dimethylthiazole-2-

yl)2,5-diphenyl-tetrazolium bromide](MTT) assay. This assay is based on the observation that the tetrazolium salt, MTT, is actively absorbed into cells and is reduced in a mitochondrial-dependent reaction to yield a formazan product. This product accumulates in the cells and can not pass through the cell membrane. The ability of cells to reduce MTT, as an indication of mitochondrial integrity and activity, is interpreted as a measure of cell viability [126].

A cationic liposome combination of dimethyl-dioctadecyl-ammonium bromide(DDAB) and dioleoyl-phosphatidyl-ethanol-amine(DOPE) has been shown to have a higher efficiency compared to other lipids. The DDAB/DOPE lipid combination increased ODN delivery in Caski cell culture 4.5 fold, compared with a 2.0-2.5 fold increase by commercially available N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethyl-ammonium-methyl-sulfate (DOTAP)[121]. In this study, we used the DDAB/DOPE cationic liposome combination as a delivery system for ASODN and evaluated its cellular uptake characteristics. We also compared the uptake efficiency of ASODN with previously used phosphatidylcholine: cholesterol liposomes. The effect of ASODN on AGT mRNA and protein was also studied in rat H-4-II-E hepatoma cell culture, a cell line known to express AGT mRNA and secrete AGT protein constitutively[127]. The effect of a control, scrambled ASODN also was determined to test the specificity of the target ASODN sequence.

Material and Methods

To determine the effects of liposome composition on the cellular uptake of ODN, H-4-II E cells were incubated in a medium containing 1 μM FITC-ODN complexed with cationic liposomes composed of DDAB(25 mg/ml) and DOPE, at different weight ratios, for 4 hours. Cells were then washed, lysed and the cellular associated fluorescence was measured by fluorometric analysis.

The effect of cationic lipid to ODN charge ratio on the cellular uptake of ODN also was determined in H-4-II E cells. The cells were incubated in a medium containing 1 μM FITC-ODN complexed with cationic liposomes with DDAB to ODN at different charge ratios for 4 hours. The cells were then lysed and the cellular associated fluorescence was measured by fluorometric analysis.

The concentration effects of ASODN with the delivery systems on ASODN uptake also were determined. H4 hepatoma cell cultures were grown to confluence, then treated with either cationic liposome-complexed ASODN, PC:cholesterol liposome-encapsulated ASODN, or naked ASODN at the following concentrations: 0.1, 0.5, 1.0, 2.5 and 5.0 μM. Cultures were incubated for 4 hours, then media was decanted and cells were lysed and assayed for FITC intensity using fluorimetric analysis.

The effectiveness of the delivery mechanism on the rate of ASODN cellular uptake in Hepatoma H4 cell culture was

determined. The cell cultures were grown to confluence and treated with either a 1 μM cationic liposome-complexed ASODN, a 1 μM PC:cholesterol liposome-encapsulated ASODN, or an 1 μM naked ASODN. Cultures were incubated for 1-4 hours. The cellular associated fluorescence was measured at 0.5, 1, 2 and 4 hours.

FITC conjugated ASODN distribution and uptake was analyzed microscopically. Hepatoma cells were grown on glass microscope slides and treated with either 1 μM of naked FITC-ASODN, 1 μM PC:cholesterol liposome-encapsulated ASODN or 1 μM cationic liposome-complexed ASODN for four hours. The slides were then mounted and the image was observed under a Nikon Optiphot-2-Fluorescent laser scanning confocal microscope.

The *in vitro* effects of cationic liposome complexed with ASODN on AGT mRNA expression and AGT protein production were determined in hepatoma cells, which were grown to confluence. These cells were treated with either 1 μM cationic liposomes complexed with ASODN, naked ASODN, scrambled ODN or cationic lipids alone. Cultures were incubated for 24 hours, then the medium analyzed for AGT by radioimmunoassay. Hepatoma cells were lysed for mRNA measurement by Northern blot analysis.

Hepatoma cells were treated with complexed ASODN at concentrations of 10 nM, 50 nM, 100 nM, 500 nM, and 1 μM to

determine the dose response effect of cationic liposome-complexed ASODN on the expression of AGT mRNA and protein. Cells were incubated with CA/ASODNs and cationic lipids used in the formulation for 24 hours, then the AGT mRNA were measured by Northern blot analysis and the AGT protein levels in the medium were measured by radioimmunoassay.

Cellular toxicity was measured using the MTT test as described in Chapter 2. Cells were incubated for 48 hours in medium containing either ASODN at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 μM, ASODN complexed with cationic lipids or the corresponding amount of cationic lipids used to complex ASODN at each concentration. After treatment, cells were washed with phosphate buffer and incubated in 0.25 mg/ml of MTT for 4 hours at 37°C. A 1 ml solution of 0.04 N HCl, prepared in isopropanol, was added and agitated for 5 minutes to solubilize the formazan produced. The absorbance was measured at 560 nm by microplate reader.

Results

Figure 3-1 summarizes the effects of DDAB to DOPE molar ratio from 1:8 to 8:1 on the cellular uptake of FITC labeled ASODN in H4 Hepatoma cells. The results demonstrate that without DOPE, ASODN uptake is low, as depicted by the 3.4±0.4% of total fluorescence detected in these cells. As the DOPE composition of the liposomes increased, cellular uptake of ASODN also increased. Maximum ASODN uptake observed

was 15.8 ±1.6%($P<0.05$) at a DDAB to DOPE weight ratio of 1:2. As more DOPE was added to the liposomes, the ASODN uptake dropped rapidly. At a weight ratio of 1:8, the ASODN uptake was 3.2±0.7%. The optimal +/- charge ratio of DDAB to ASODN on the cellular uptake of ASODN was determined by treating the cells with 1μM of FITC-ASODN complexed with DDAB at different molar ratios. The uptake of ASODN complexed with liposomes composed of DDAB alone was used to test the effect of the helper lipid DOPE on the uptake of ASODN. Figure 3-2 shows that the maximum uptake was 16.1±1.9%($P<0.05$) at a DDAB to ODN molar ratio of 5:1. Without DOPE, the ASODN uptake was low, ranging from 2.6±0.2% to 3.7±0.5%.

In a subsequent study with hepatoma cells, the time course of cellular uptake was evaluated with all three delivery Systems (naked ASODN, PC:cholesterol-encapsulated, and cationic liposome-complexed ASODN) using a 1 μM concentration of ASODN. Cellular uptake was determined at given time intervals after cells were treated with FITC labeled ASODN.

Figure 3-3 demonstrates that cationic liposome complexation resulted in a more rapid cellular uptake of ASODN, with greater ASODN accumulation per time point when compared to both the PC:cholesterol liposome treated cells and cells treated with naked ASODN. With all treatments the most rapid intracellular ASODN accumulation was observed between 0 and 60 minutes after ASODN administration.

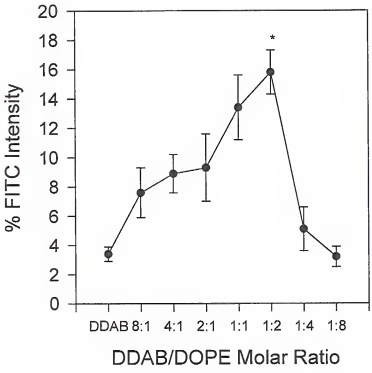


Figure 3-1. Effect of DDAB to DOPE molar ratio on the cellular uptake of FITC-labeled ASODN. Hepatoma cells were incubated with 1 μM of ASODN and DDAB (25 mg/ml) with DOPE at different molar ratios for 4 hours. The cellular associated FITC intensity was then measured by fluorimetry. Data represents mean ± standard error (n=3) $P<0.05$(*).

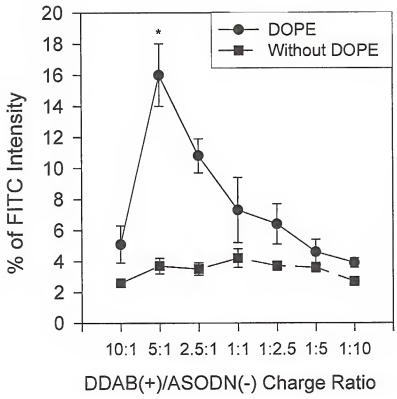


Figure 3-2. Effect of DDAB to ASODN charge ratio on the cellular uptake of FITC-labeled ASODN. Hepatoma cells were incubated with 1 μM ASODN with DDAB at a charge ratio of 10:1 to 1:10 in the presence or absence of DOPE for 4 hours. The cellular associated FITC intensity was measured by fluorimetry. Data represents mean ± standard error (n=3) $P<0.05$(*).

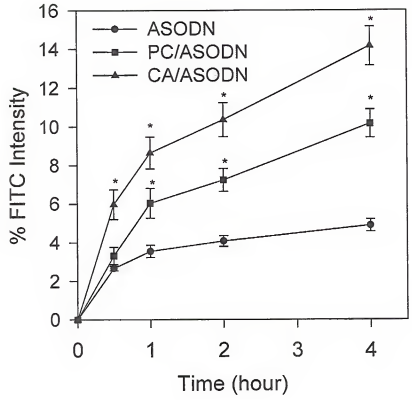


Figure 3-3. Time course of cellular uptake of FITC- labeled ASODN. Hepatoma cells were incubated with 1 μM of ASODN, PC cholesterol liposome-encapsulated ASODN(PC/ASODN), or cationic liposome complexed ASODN(CA/ASODN) for 0.5 to 4 hours, then the cellular associated FITC intensity was measured by fluorimetry. Data represents mean ± standard error(n=3) $P<0.05$(*).

Figure 3-4 summarizes the results of the cellular-associated fluorescence intensities associated with FITC-labeled ASODN uptake for a range of concentrations using each delivery mechanism. These data demonstrate that the amount of ASODN within the cells increased with each concentration. The amount of cellular ASODN accumulated was greater ($P<0.05$) with the PC:cholesterol liposome ASODN encapsulation delivery system compared to that observed with the naked ASODN at all the measured ASODN concentrations. However, this accumulation was most profound with the cationic lipid delivery. With cationic lipid delivery, the accumulation of ASODN was nearly linear at low concentrations; however, the amount of ASODN taken up did not increase substantially at concentrations greater than 1 μM. Also, the 4-hour treatment with 1μM concentration depicted in Figure 3-3 demonstrated results similar to those observed with the 1 μM concentration in Fig 3-4, signifying the reproducibility of the system.

Four hours after treatment, the cellular uptake and distribution of FITC-labeled ASODN was observed with confocal microscopy. Figure 3-5A shows a weak intracellular background fluorescence of untreated cells. Figure 3-5B shows the distribution of naked FITC-labeled ASODN. Although some intense fluorescence is observed within the cells, there appears to be a large proportion of fluorescence localized at the cell membrane surrounding the individual cells. Figure 3-5C shows cells treated with PC:cholesterol-encapsulated FITC-

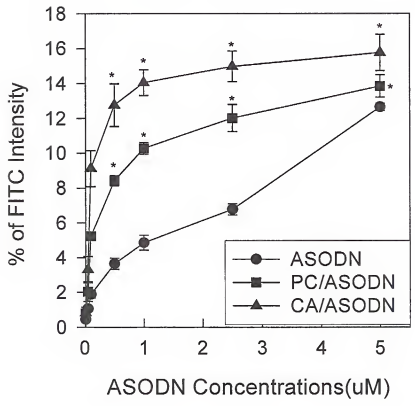


Figure 3-4. Dose-dependent cellular uptake of FITC-labeled ASODN. Hepatoma cells were incubated with 1 μM of ASODN, PC cholesterol liposome-encapsulated ASODN(PC/ASODN), and cationic liposome-complexed ASODN(CA/ASODN) at ASODN doses from 100 nm to 5 μM for 4 hours. The cellular associated FITC intensity was then measured by fluorimetry. Data represents mean ± standard error (n=3) $P<0.05$(*).

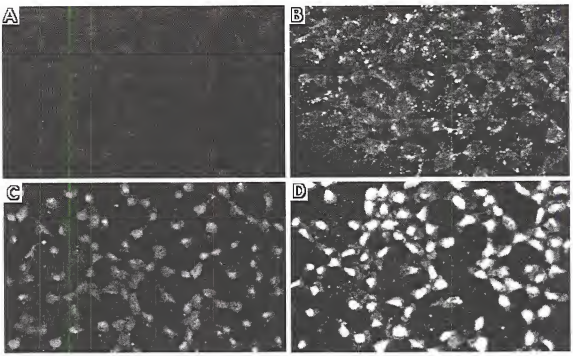
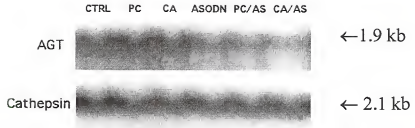


Figure 3-5. Cellular uptake and intracellular distribution of FITC-labeled ASODN observed by confocal microscopy. Hepatoma cells were grown on microslides then incubated with 1 μM ASODN, PC cholesterol liposome encapsulated ASODN (PC/ASODN), or cationic liposome complexed ASODN(CA/ASODN) for 4 hours. The cells were then fixed and the images were observed by confocal microscopy; A. control; B. cells treated with ASODN; D. cells treated with PC/ASODN; D. cells treated with CA/ASODN (n=3).

labeled ASODN. Most of these cells appear to have a fairly even intracellular distribution of ASODN, with very little of ASODN remaining outside the cell. The fluorescence was stronger within these cells compared to that observed after naked ASODN treatment. In some cells there was an accumulation of fluorescence in localized cellular areas, which may indicate nuclear accumulation. Finally, Figure 3-5D shows a more intense localized fluorescence observed in cells treated with cationic liposome-complexed, FITC-labeled ASODN. The fluorescence within these cells was the strongest compared to all other treatments and appeared to be mainly localized within the cell nucleus.

Figure 3-6a demonstrates the AGT and control cathepsin D mRNA bands after various treatments measured by the Northern blot analysis. Figure 3-6b displays the density ratio of AGT to cathepsin D mRNA. In cell culture, the amount of AGT mRNA was shown to be decreased 75% after treatment with the cationic liposome-complexed ASODN, compared with a 30% decrease after naked ODN treatment. The control scrambled ODN and cationic lipid had no effect on mRNA expression and was similar to the untreated control. AGT protein production was attenuated similarly, and a more pronounced decrease was observed in those samples treated with cationic liposome-complexed ASODN (Figure 3-7). In untreated control cells the baseline AGT level was 52.0±2.46 ng/ml. There was no significant decrease in AGT production from baseline levels in the lipid and complexed scrambled control groups, which

a.



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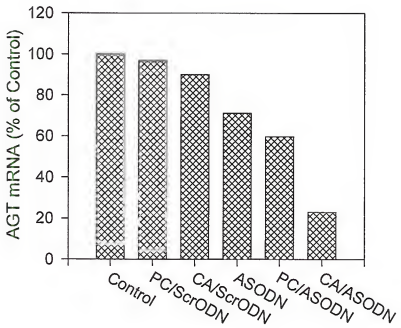


Figure 3-6. Effect of liposome-associated ASODN on AGT mRNA expression in hepatoma cell culture. Cells were incubated with 1 μM of ASODN, PC/cholesterol liposome-encapsulated ASODN(PC/ASODN), cationic liposome-complexed ASODN (CA/ASODN), PC/cholesterol liposome encapsulated scrambled ODN(PC/ScrODN) or cationic liposome complexed scrambled ODN(CA/ScrODN) for 24 hours. AGT mRNA was measured by Northern blot analysis: a. the result of Northern blot hybridization for AGT (1.9 kb) and the control gene cathepsin D(2.1 kb); b. the density ratio of AGT to cathepsin.

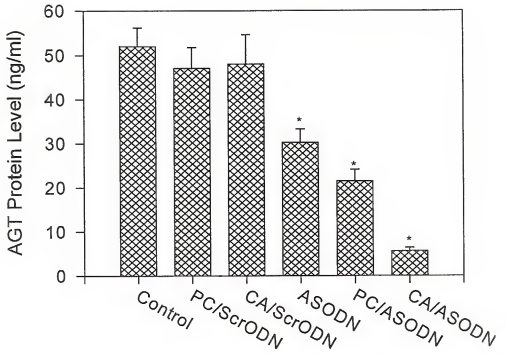


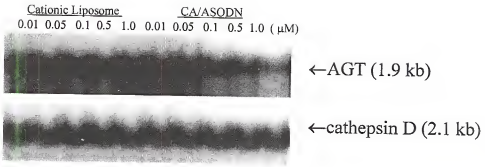
Figure 3-7. Effect of liposome-associated ASODN on AGT protein expression in hepatoma cell culture. Cells were incubated with 1 μM of ASODN, PC/cholesterol liposome encapsulated ASODN(PC/ASODN), cationic liposome complexed ASODN(CA/ASODN), PC/cholesterol liposome encapsulated scrambled ODN(PC/ScrODN) or cationic liposome complexed scrambled ODN(CA/ScrODN) for 24 hours. AGT protein levels were measured by radioimmunoassay. Data represents the mean ± standard error (n=3) $P<0.05$(*).

were 45.53±5.9, and 47.03±6.9 ng/ml, (n=3), respectively. In cells treated with naked ASODN, PC/ASODN and cationic liposome complexed ASODN, AGT levels were significantly ($P<0.05$) decreased from baseline levels: 30.2±3.0, 22.1±2.7, 5.61±0.95 ng/ml, respectively.

The dose response effect of CA/ASODN treatment demonstrates that AGT mRNA was reduced at an ASODN concentration of 100 nM. Northern blot analysis revealed that at both 100 and 500 nM concentrations of ASODN, there was a 40% reduction in AGT mRNA. At a higher concentration of 1 μM, a 70% decrease in AGT mRNA was observed(Figure 3-8). The decrease in AGT protein(Figure 3-9) at an ASODN concentration of 50 nM was 40.29±4.0 ng/ml compared to the 51.77±3.7 ng/ml observed in the control samples. At 0.1, 0.5 and 1 μM ASODN, AGT protein levels were significantly ($P<0.05$) decreased from the baseline in a dose dependent manner, with AGT concentrations of 26.68±5.3; 23.27±6.1; 5.67±0.3 ng/ml, respectively. Cationic liposomes alone had no effect on AGT protein or mRNA levels.

ASODN cytotoxicity was determined over the concentration ranges of 0.1 to 10 μM. The toxicity of the CA/ASODN complexes and the cationic lipids used to complex the ASODN also was determined. Figure 3-10 demonstrates that 89% of the cells survived at a naked ASODN concentration of 10 μM, while

a.



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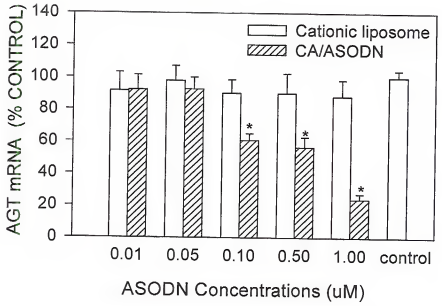


Figure 3-8. Dose-dependent effects of CA/ASODN on AGT mRNA in hepatoma cell culture. Cells were incubated with CA/ASODN at doses from 10 nM to 1 μM for 24 hours. AGT mRNA was measured by Northern blot analysis: a. the result of Northern blot hybridization for AGT(1.9 kb) and the control gene cathepsin D(2.1 kb); b. the relative intensity of AGT gene expression compared with control(n=3)(*)$P<0.05$.

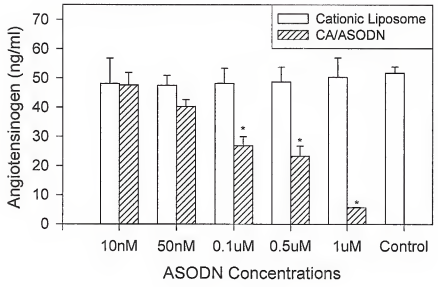


Figure 3-9. Dose-dependent effects of CA/ASODN on AGT protein expression in hepatoma cell culture. Cells were incubated with CA/ASODN at doses from 10 nM to 1 μM for 24 hours. AGT protein levels were measured by radioimmunoassay. Data represents mean ± standard error (n=3) $P<0.05$(*).

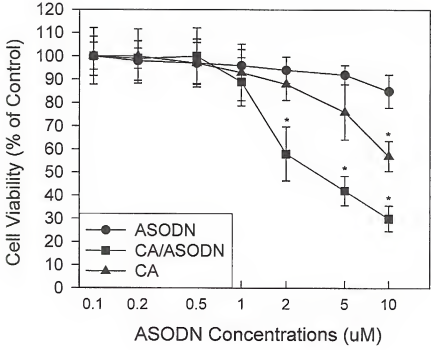


Figure 3-10. In vitro cytotoxicity of CA/ASOSN, ASODN and cationic lipids measured by MTT test. Hepatoma cells were treated with ASODN, cationic lipids(CA), or cationic liposome complexed ASODN(CA/ASODN) at ASODN concentrations from 100 nM to 10 μM for 48 hours, then cell viability was measured by MTT test. Data represents mean ± standard error (n=3)P<0.05(*).

at lower concentrations no obvious toxic effect was observed. The ASODN lipid complex showed a similar cytotoxic effect at ASODN concentrations up to 0.5μM. Toxicity appeared to increase as ASODN concentration increased. At an ASODN concentration of 10 μM the complex caused approximately 70% of the cells to die. The cationic lipids seemed less toxic than the complex. At an ASODN concentration of 0.5 μM, the toxic effect was similar to that of CA/ASODN. However, at a concentration of 10 μM, 50% of the cells survived. The toxicity of the CA/ASODN complexes at 1.0 μM concentrations was seen to be similar to the toxicity of naked ASODN at the 10 μM concentration in the Hepatoma cell culture.

Discussion

Successful attenuation of the target protein has been achieved using antisense technology in a wide range of biological systems[70-72]. However, the development of antisense therapy has not been as smooth as once anticipated. Problems have been encountered in the delivery of the molecules to target sites, with general uptake efficiency of naked ASODN being as low as 2%[140]. The cationic liposomes used in this study were shown to improve the cellular uptake of ASODN in hepatoma cell culture. The composition of the lipids and the charge ratio of cationic lipid to ASODN appeared to be critical factors for successful transfection

(Figure 3-1,3-2). The uptake of the ASODN dramatically changed under different lipid to ASODN charge ratios. This is probably due to such factors as aggregation of the cationic lipids with the ASODN, the size of the complex particle and the overall charge of the ASODN-lipid complexes at different charge ratios. The uptake of ASODN is relatively higher when the complex has a net positive charge than when the complex has an overall net negative charge. The net positive charge may provide stronger forces of interaction between the complex and the negatively charged cell membrane. Inclusion of DOPE in the liposome appears to be essential for efficient delivery due to its fusogenic function[101,132].

Results from the scanning confocal microscope (Figure 3-5) demonstrated FITC labeled ASODN is distributed intracellularly. The presence of fluorescence in the cytoplasm in a punctuated manner is considered to be consistent with distribution within endocytic vesicles, suggesting that cellular uptake of ASODN may be via an endocytotic process. In cells treated with cationic liposome-complexed ASODN, the fluorescence signal seemed diffuse in the cytoplasm and in the nucleus, suggesting that cationic liposomes may function to facilitate the release of ASODN from the endosomal vesicles. The extent of the endocytotic processes on the uptake of ASODN has been studied using different inhibitors of endocytosis. Agents that increase lysosomal pH, such as chloroquine and NH_4Cl, did not prevent accumulation of ASODN within the nucleus or block ASODN activity[141]. These

observations suggest the participation of endosomal acidification in the release of ODN from the endosome into the cytoplasm.

The cationic liposomes also were shown to increase the rate and the amount of ASODN accumulation by cells (Figure 3-3,3-4). At the beginning of the incubation, CA/ASODN displayed a higher association with the cells than either the naked ASODN or the PC/ASODN. It has been suggested that the initial stage of CA/ASODN interaction with cells induces adsorption of the positively charged CA/ASODN complexes to the negatively charged phospholipids of the cell membrane. The charge-induced interaction may account for the enhanced cellular association of CA/ASODN, compared with the PC/ASODN and naked ASODNs. The results from the time and dose-dependent uptake curves suggest that ASODN uptake may be a saturable process. ASODN uptake reached a plateau at the four-hour time point at a 1 uM concentration.

Subsequent studies evaluated the effect of ASODN on AGT gene expression. The results demonstrate that CA/ASODN decreases AGT mRNA and protein in Hepatoma cell culture. The possible mechanisms for ASODN inhibition of gene expression may be explained as ASODN functioning as a "road blocker" to ribosome assembly thus impeding the binding of translation factors or acting to block translocation of ribosome on the mRNA. The involvement of RNase H, which hydrolyses the RNA part of RNA/DNA hybrids, may also be a possible mechanism. RNase H is a ubiquitous enzyme, with varying activity among

different cell types[75]. The binding of ASODN to targeted mRNA may activate RNase H, which then degrades the RNA portion of an RNA-DNA duplex, and subsequently blocks gene expression. The RNase H mechanism is supported by the fact that failed translation blockage was observed in rabbit reticulocyte lysate which contains low to no RNase H activity[142]. The role of RNase H also is demonstrated by using an ASODN that is known to not activate RNase. The oligonucleotides such as methyl phosphonate and α-oligomers targeted to the coding region of the rabbit β-globin mRNA did not affect β-globin synthesis in wheat germ extract nor Xenopus oocytes[143].

These results also demonstrated that the effects of ASODN on the expression of AGT mRNA and protein levels was significantly enhanced by cationic liposomes, probably due to increased cellular and nuclear delivery of ASODN into the cells. Cationic liposomes also may protect the ASODN from enzyme degradation in the culture medium. The reported half-life of phosphorothioated ASODN in the cell culture medium RPMI 1640, with 10% fetal bovine serum, undiluted fetal bovine serum or rat cerebrospinal fluid is 14±2 hour, 8±1 hours and 19±7 hours, respectively[144]. Cationic liposome complexation also has been shown to increase the stability of ASODN in cell culture[145].

The cytotoxic effect of the cationic liposome ASODN complexes appears higher than either the ASODN molecules or

the cationic lipids alone (Figure 3-10). The toxic effects of cationic lipid are attributed to their surfactant like properties which cause solublization and poration of the cell membrane with subsequent damage to the cell integrity at higher concentrations[139]. The higher toxicity of CA/ASODN complexes may be a result of the non-specific effects of ASODN. Cationic liposomes resulted in increased intracellular delivery of ASODN. At high concentrations, ASODN may bind to non-targeted sequences and cause degradation of proteins, which may be essential for cell viability. This result was consistent with the previous *in vitro* transcription/ translation experiments, which showed that the scrambled ODNs also decreased the expression of AGT mRNA at ASODN concentrations higher than 3μM[68]. The naked ASODN showed minimal cytotoxicity, probably due to its short half-life in the culture medium. Cationic liposome complexation improves its stability, but also increases its toxic effect. However, when used at doses of 1 μM or less, toxic effects are minimized, and are equivalent to the toxicity produced by 10 times the concentration of ASODN alone.

To summarize, these results support the following conclusions. Cationic liposomes consisting of DDAB and DOPE increase the cellular delivery of ASODN, compared with previously used PC-cholesterol liposomes in hepatoma cell culture. Cationic liposome-complexed ASODN targeted to AGT mRNA decreased AGT mRNA and protein in cell culture in a dose

dependent manner. The CA/ASODN complex appeared to have a higher toxicity than either the cationic lipids or the ASODN alone, possibly due to the non-specific effects of high intracellular concentrations of ASODN.

CHAPTER 4

THE EFFECT OF ROUTE OF ADMINISTRATION OF CA/ASODN ON BLOOD PRESSURE AND TISSUE DISTRIBUTION OF ASODN IN SHR: IMPLICATIONS OF THE ROLE OF TISSUE RAS ON BLOOD PRESSURE REGULATION

Specific Aims

The specific aim of this part of the research was to develop a mechanism for targeted antisense oligonucleotide delivery to the liver using liposome technology and to determine ASODN tissue distribution after intraarterial and intravenous administration in SHR model and its subsequent effects on hypertension.

Introduction

The previous *in vitro* studies demonstrated that cationic liposomes composed of DDAB and DOPE are more effective in delivering ASODN than PC-cholesterol liposomes in hepatoma cell culture. The previous *in vivo* studies showed that PC-cholesterol encapsulated ASODN successfully decreased blood pressure when administered both centrally and intra-arterially in the SHR model of hypertension[65,68]. However, *in vivo* data was inconclusive when ASODN was administered intra-venously, despite the success of ASODN mediated gene

inhibition. Tissue and cellular ASODN delivery of ASODN remain the major obstacles for intended ASODN activity[77]. Optimization of the ASODN delivery system and route of administration have been considered the most important aspects of improving the intended biological effect of ASODN.

The role of angiotensinogen in the pathogenesis of hypertension has been supported by genetic studies[37-39]. AGT is expressed and constitutively secreted by hepatocytes. Successful delivery of ASODN to target cells should lead to suppression of AGT mRNA expression, resulting in decreased activity of the RAS and subsequent attenuation of hypertension. Delivery of macromolecules such as oligonucleotides to their target sites in vivo requires successful trans-endothelial migration and target cell endocytosis[146]. Strategies for liver(tissue)-specific targeting are divided into passive and active targeting. Passive targeting refers to the utilization of the natural disposition profiles of a drug carrier, which is determined by the physiochemical properties of the chemicals relative to the anatomical and physiological characteristics of the body. Delivery of macromolecules to the liver reticuloendothelial system, which lacks basement membrane on the endothelial cells and allows molecules 100 nm or less in diameter to permeate through, is an example of this. Active targeting refers to the alterations of the natural disposition of a drug carrier in order to direct it to

specific cells, tissues, or organs. Ligands, or monoclonal antibodies, which can bind specifically to the surface of target cells are used for this purpose[147]. In the case of hepatic targeting, antibodies targeted to asialoglycoprotein receptors, which are uniquely expressed by the liver, have been used to improve liver targeting[148,149].

In this study, we utilized a cationic liposome approach to enhance ASODN delivery to the liver target. Small liposomes with a diameter of less than 100 nm are known to be naturally and rapidly cleared by the liver reticuloendothelial system after injection[96]. Compared with previously used PC-cholesterol liposomes, positively charged cationic lipids may facilitate the adsorption of liposome/ASODN complex to the negatively charged cell surface and subsequently increase the cellular delivery of ASODN molecules. Incorporating the pH-sensitive fusogenic lipid DOPE also may facilitate target cell endocytosis of ASODN[150,151].

The renin-angiotensin system(RAS) has long been known as a circulating endocrine system that regulates blood pressure and fluid and electrolyte balance via its effector peptide angiotensin II[8]. The colocalization of renin, ACE, and angiotensin II receptor messenger RNA in tissues such as the kidney, heart, and brain suggest the existence of local RASs, which may play a functional role in blood pressure regulation [11-14]. Unlike the hormonal RAS, which regulates blood pressure by a closed-loop negative feedback mechanism,

the local RAS is thought to function in a paracrine-autocrine manner. Angiotensin II produced by synthesizing cells can act on receptors of the neighboring cells (paracrine), or act on the receptors of the cells where it was synthesized (autocrine) to regulate such functions as smooth muscle cell contraction or release of endothelium derived relaxing factors[17-19].

The involvement of the tissue RAS in blood pressure regulation was first suggested by the effect of anti-hypertensive drugs. ACE and renin inhibitors with different physicochemical properties, and thus different tissue penetration profiles, showed different antihypertensive effects[11]. The antihypertensive effects of ACE inhibitors and their duration are more consistent with the inhibition of ACE activity in the kidney and aorta rather than the plasma [152]. Renin inhibitors also appear to lower blood pressure in a fashion dissociated from their effect on plasma renin[153].

An intrarenal RAS has also been proposed as individual components of the RAS were detected in the renal cortex [154]. At a local level, angiotensin II influences glomerular micro-circulation, causing reductions in plasma flow rate; the ultrafiltration coefficient; and increases in the hydrostatic pressure difference and renal arteriolar resistance.

The SHR essential hypertension animal model is inbred from the normotensive strain of WKY rat[61]. Abnormal RAS

activity resulting from genetic selection is believed to be responsible for the high blood pressure in SHRs[155]. In this study, we determined the effect of ASODN on blood pressure 24 hours after injection in both SHR and WKY strains, to investigate the role of the RAS in hypertension.

In previous studies, we measured the effect of blood pressure changes after intra-arterial(IA) and intravenous (IV) administration of ASODN in SHR. It appeared that ASODN caused more pronounced decreases in blood pressure after IA injection of ASODN than after IV injection. The discrepancy between blood pressure changes could result from tissue specific effects as a consequence of different tissue distribution of ASODN after IA and IV injection. In this study, the effects of intraarterial and intravenous administered ASODN on blood pressure, tissue distribution of ASODN, plasma and tissue angiotensinogen levels, were measured.

Material and Methods

To determine the mean arterial pressure (MAP) changes in SHR and WKY rats after intraarterial and intravenous injection of cationic liposome complexed ASODN, groups of rats (250-275 g, n=6), a catheter was implanted in the carotid artery and for intravenous studies, the femoral vein. Rats were allowed to recover for 24 hours after surgery, then baseline mean arterial blood pressure was determined using direct blood pressure measurement. A catheter was inserted into the external carotid artery and

connected to a pressure transducer that was interfaced with a Digi-Med BP Analyzer(micro-Med, Indianapolis,Ind). Signals were recorded on a Gould TA2405 EasyGraf Physiograph, which provides information on systolic, diastolic, and mean arterial pressure and heart rate. Following baseline pressure measurements, 50 μg doses of either cationic liposome complexed ASODN, scrambled ODN, uncomplexed ASODN or cationic lipid were injected either IA via the carotid catheters or IV via the femoral catheters. Twenty-four hours after injection, mean arterial pressure was measured using the same method.

The tissue distribution of FITC conjugated ASODN and cationic liposome complexed FITC-ASODN after intravenous and intraarterial administration was then determined. Groups of Sprague Dawley rats(n=9) were injected with 100 μg FITC-ASODN and FITC-CA/ASODN via the carotid artery or femoral vein. One, 8, and 24 hours later, three of the injected rats were perfused with saline and then decapitated. Liver, kidney, heart, lung, brain, and plasma were collected. Tissues were then homogenized in Triton 100 and the associated fluorescence intensity was measured using fluorimetric analysis.

We also measured plasma AGT and Ang II levels after intraarterial and intravenous administration of ASODN. 50 μg of cationic liposome complexed ASODN was injected via the carotid artery or femoral vein of SHR(n=3). After 24 hours, animals were decapitated and plasma samples were collected.

Plasma AGT and Ang II(RK-A22, Alpco, Windham, NH) were measured using radioimmunoassay[124].

In order to determine the effects of ASODN on tissue AGT expression, 50 μg doses of either cationic liposome complexed ASODN, scrambled ODN, uncomplexed ASODN or cationic lipid were injected either IA or IV in SHRs(n=3). After 24 hours, rats were sacrificed and liver, kidney, and heart were collected. Total mRNA was extracted and AGT and Cathepsin D mRNA levels were measured by Northern blot analysis[122,123]. The house-keeping gene cathepsin D was used as a control to ensure equal loading of RNA.

Results

Figure 4-1 demonstrates the change in mean arterial pressure 24 hours after intraarterial injection of either CA/ASODN, CA/ScrODN, uncomplexed ASODN, or cationic lipids. A significant ($P<0.05$) decrease in MAP, 23±5 mmHg from baseline, was observed in SHR treated with CA/ASODN. A less marked but significant($P<0.05$) decrease in blood pressure, 15±4 mmHg from baseline level, was observed in SHRs treated with uncomplexed ASODN. Blood pressure were unchanged in animals treated with either CA/ScrODN or cationic lipids (Figure 4-1). ASODNs administered by the intravenous route produced a significant but smaller decrease in blood pressure: 7±2 mmHg and 4±1 mmHg($P<0.05$) from baseline after either CA/ASODN or ASODN treatment, respectively (Figure 4-2). No significant changes in blood pressure were observed

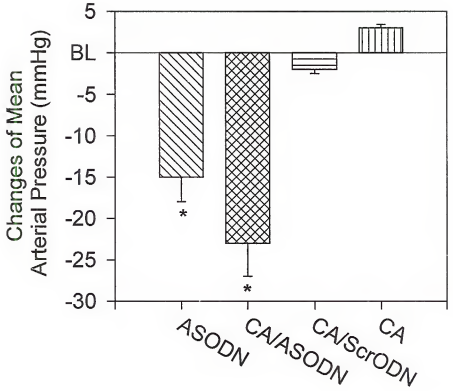


Figure 4-1. Mean arterial pressure changes from baseline 24 hours after intra-arterial injection of CA/ASODN in SHR. 50 μg cationic liposome-complexed ASODN(CA/ASODN), ASODN, cationic lipids (CA), and cationic liposome-complexed scrambled ODN (CA/ScrODN) were used. Blood pressure was measured by direct method. Data represent mean ± standard error (n=6). $P<0.05$(*).

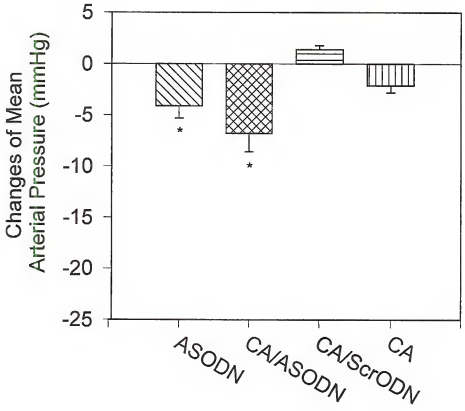


Figure 4-2. Mean arterial pressure changes from baseline 24 hours after intravenous injection of CA/ASODN in SHR. 50 μg cationic liposome-complexed ASODN(CA/ASODN), ASODN, cationic lipids(CA), and cationic liposome-complexed scrambled ODN(ScrODN) were used. Blood pressure was measured by direct method. Data represents mean ± standard error(n=6). $P<0.05$(*).

after CA/ScrODN or cationic lipid treatment. Similar experiments also were conducted in control WKY rats and no significant blood pressure changes from baseline level, 132± 8 mmHg, were observed after IA or IV injection of ASODNs or controls.

To determine the mechanism of the observed differences in blood pressure reductions after IA and IV injection, tissue distribution of FITC labeled ASODN after IA and IV injection was determined. Figures 4-3, 4-4, and 4-5 summarize the measured tissue associated FITC intensity for each injected dose at 1, 8, and 24 hours. Figure 4-3 demonstrates that at the 1 hour time point, 30% of the injected complexed ASODN accumulated in the liver after IA injection, compared with 25% accumulation in the liver after IV injection. Cationic liposome complexation increased ASODN accumulation in the liver, lung, and heart by approximately 100%. As expected, a higher accumulation of CA/ASODN was seen in the lung after IV injection than after IA injection. At the 8 hour time point, a similar ASODN distribution was observed with either route of administration(Figure 4-4). At the 24-hour time point, a similar degree of CA/ASODN accumulation was observed in the liver after IA and IV injection. However, in the kidney, there was a greater accumulation of CA/ASODN after IA injection than after IV injection. With time the brain appeared to have greater level of accumulation.

Plasma AGT levels of SHR, 24 hours after IA injection of cationic lipids, CA/ScrODN, CA/ASODN, or ASODN, were 117±15

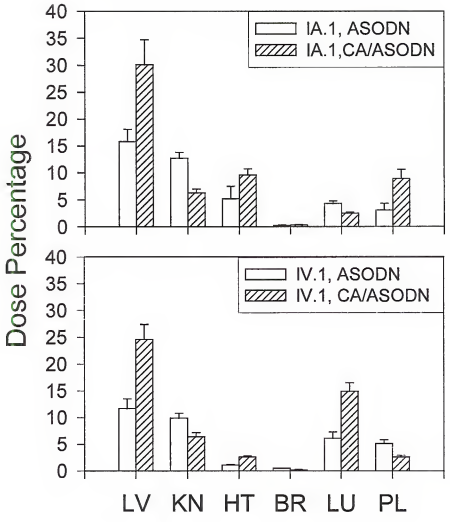


Figure 4-3. Tissue distribution of ASODN and CA/ASODN in 1 hour after IA or IV injection. The FITC intensity were measured in liver(LV), heart(HT), kidney(KN), lung(LU), brain(BR), and plasma(PL) in Sprague Dawley rats. Figure demonstrates the FITC intensity measured by fluorimetry as a percentage of the injected dose. Data represent mean ± standard error(n=3). $P<0.05$(*).

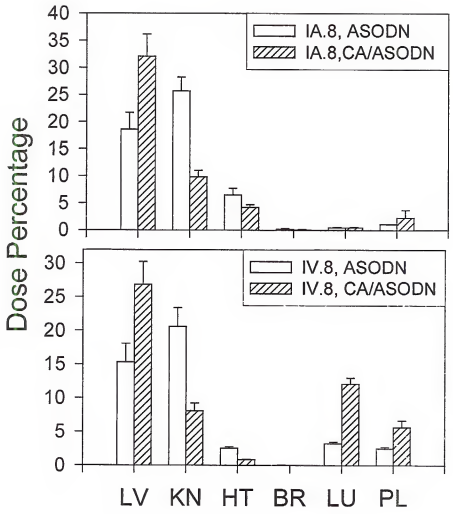


Figure 4-4. Tissue distribution of ASODN and CA/ASODN in 8 hour after IA or IV injection. The FITC intensity were measured in liver(LV), heart(HT), kidney(KN), lung(LU), brain(BR), and plasma(PL) in Sprague Dawley rats. Figure demonstrates the FITC intensity measured by fluorimetry as a percentage of the injected dose. Data represent mean ± standard error(n=3). $P<0.05$(*).

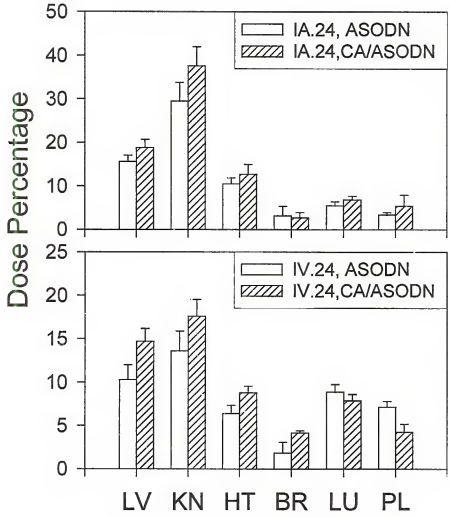


Figure 4-5. Tissue distribution of ASODN and CA/ASODN in 24 hour after IA or IV injection. The FITC intensity were measured in liver(LV), heart(HT), kidney(KN), lung(LU), brain(BR), and plasma(PL) in Sprague Dawley rats. Figure demonstrates the FITC intensity measured by fluorimetry as a percentage of the injected dose. Data represent mean ± standard error(n=3). $P<0.05$(*).

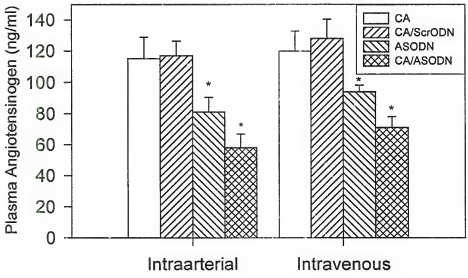


Figure 4-6. Plasma AGT levels 24 hours after IA and IV injection of ASODN with liposomes in SHR. Angiotensinogen was measured by radioimmunoassay. Data represents mean ± standard error (n=3). $P<0.05$(*).

ng/ml, 119±10 ng/ml, 46±10 ng/ml, and 69±12 ng/ml, respectively. After IV injection, the levels were 120±14 ng/ml, 128±14 ng/ml, 58±9 ng/ml, and 71±7 ng/ml, respectively (Figure 4-6). The plasma levels of AGT decreased significantly($P<0.05$) after either CA/ASODN or ASODN treatment, compared with CA/ScrODN or cationic lipid treatment. However, no significant difference of AGT levels between IA or IV injection of either CA/ASODN or ASODN was observed.

Similar reductions of plasma angiotensin II levels were also observed. Figure 4-7 summarizes the effect of CA/ASODN treatment on plasma angiotensin II levels in SHR. Ang II levels were 208±26 ng/ml, 226±16 ng/ml, 81±8 ng/ml, and 160± 9 ng/ml after IA injection of cationic lipids, CA/ScrODN, CA/ASODN, or ASODN, respectively. After IV administration, the levels were 212±18 ng/ml, 209±9 ng/ml, 94±10 ng/ml, and 172±12 ng/ml, respectively. The Ang II levels after either CA/ASODN or ASODN treatment were significantly ($P<0.05$) lower compared with that after ScrODN and CA treatment. The difference between angiotensin II levels after IA and IV injection was not statistically significant.

We also measured AGT mRNA levels in the heart, kidney and liver after IA and IV injection of ASODNs using Northern blot analysis. In the kidney, AGT mRNA decreased approximately 50% 24 hours after IA ASODN administration, compared with a 20-30% decrease observed after IV injection

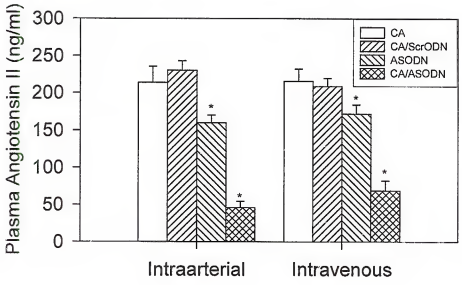
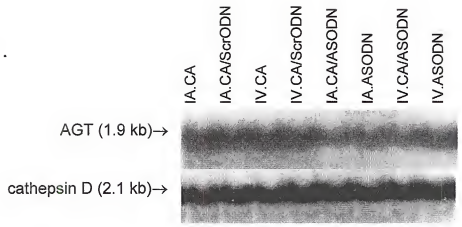


Figure 4-7. Plasma angiotensin II levels 24 hours after IA or IV injection of ASODN with liposomes in SHR. Angiotensin II was measured by radioimmunoassay. Data represents mean ± standard error (n=3). $P<0.05$(*).

a.



b.

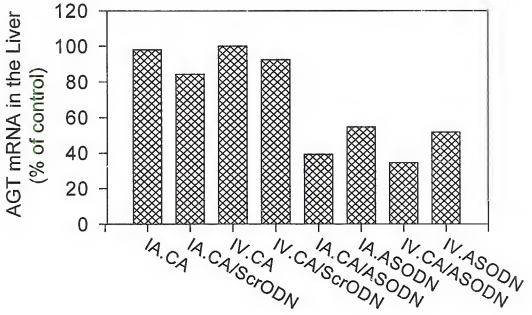
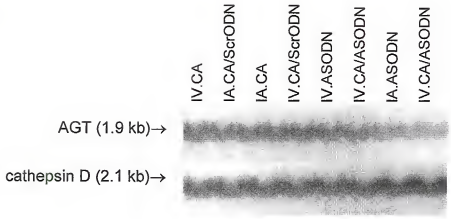


Figure 4-8. Liver AGT mRNA levels in SHR 24 hours after IA or IV injection of ASODN with liposomes. 50 ug of cationic liposome-complexed ASODN (CA/ASODN), cationic liposome-complexed ScrODN (CA/ScrODN), cationic lipids(CA) or ASODN were used: a. the Northern blot analysis of AGT(1.9 kb) and control cathepsin D(2.1 kb); b. the relative values of AGT mRNA compared with control(n=3). $P<0.05$(*).

a.



b.

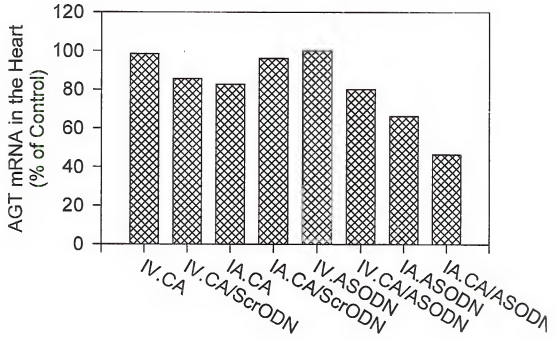
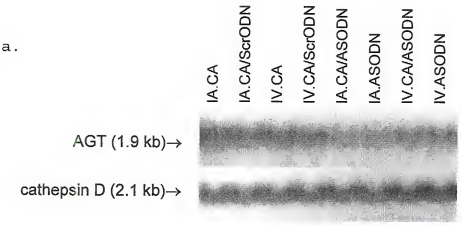


Figure 4-9. Heart AGT mRNA levels in SHR 24 hours after IA or IV injection of ASODN with liposome. 50 μg of cationic liposome-complexed ASODN (CA/ASODN), cationic liposome-complexed ScrODN (CA/ScrODN), cationic lipids(CA), or ASODN were used: a. the Northern blot analysis of AGT(1.9 kb) and control cathepsin D(2.1 kb); b. the relative values of AGT mRNA compared with control(n=3). $P<0.05$(*).

a.



b.

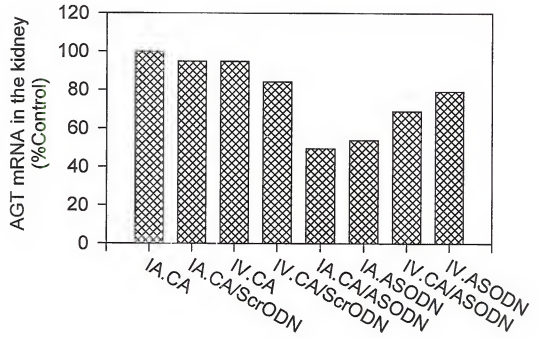


Figure 4-10. Kidney AGT mRNA levels in SHR 24 hours after IA or IV injection of of ASODN with liposomes. 50 μg of cationic liposome-complexed ASODN (CA/ASODN), cationic liposome-complexed ScrODN (CA/ScrODN), cationic lipids(CA), or ASODN: a. the Northern blot analysis of AGT (1.9 kb) and control cathepsin D(2.1 kb); b. the relative values of AGT mRNA compared with control (n=3). $P<0.05$(*).

(Figure 4-8). In the liver, AGT mRNA decreased similarly after either IA or IV injection of CA/ASODN, while larger decreases, 60%, were observed in rats treated with CA/ASODN, compared with 40% decreases after treated with ASODN alone at the 24 hour time point (Figure 4-9). In the heart, AGT mRNA decreased more after IA than after IV injection of either CA/ASODN or ASODN alone. Decreases in AGT mRNA after intraarterial CA/ASODN, ASODN and intravenous CA/ASODN treatment were 60%, 30%, 20%, respectively. No significant changes in AGT mRNA was observed after IV ASODN treatment(Figure 4-10).

Discussion

A significant decrease in blood pressure was observed 24 hours after both intraarterial and intravenous injection of cationic liposome-complexed ASODN(50 ug dose) targeted to AGT mRNA in the SHR. The decrease in blood pressure after IA injection was consistent with previous results[68]. Although it is generally considered that in the renin-angiotensin enzymatic cascade, that renin is the rate-limiting factor which determines the amount of AGT being converted to angiotensin II, while AGT is constitutively released from the hepatocytes and is always present in extra amounts for the renin reaction[38]. The results of these studies showed that inhibition of the AGT gene expression in vivo decreases blood pressure effectively, which may provide a novel therapeutic choice for treating hypertension. Our results

also imply that AGT may be involved in the pathogenesis of hypertension.

The difference in magnitude of blood pressure decreases after IA and IV administration might be attributed to the difference in tissue distribution of ASODN, resulting from different routes of administration, and subsequently leads to specific tissue-mediated effects. Compared with IA administration after IV injection, a larger part of the CA/ASODN particles are sequestered by the lungs when the circulation presents them to this organ. The accumulation of ASODN by other tissues, such as kidney and heart, may subsequently decrease due to the lowered ASODN concentration in the circulation as the ASODN is sequestered by the pulmonary system. The tissue distribution of ASODN 24 hours after injection showed a higher ASODN accumulation occurred in the lung after IV than after IA injection, while greater accumulation of ASODN occurred in the kidney after IA than after IV injection. The accumulation in the liver was slightly higher after IA than after IV injection (Figure 4-5). The difference in tissue distribution after IA and IV injection suggests that a tissue or organ effect, such as a renal effect, might be involved in the observed blood pressure decreases.

This study also showed that for both intraarterial and intravenous administration, blood pressure decreases were more pronounced after treatment with cationic liposome-complexed ASODN compared with that after the uncomplexed

ASODN treatment. This is in agreement with the hypothesis, which proposes that cationic liposome enhances the antihypertensive effect of ASODN. These observations are also consistent with the *in vitro* studies, which suggest that cationic liposomes enhance the cellular delivery of ASODN and subsequently potentiates the inhibitory effects of ASODN on AGT mRNA and protein production in cell culture. Previous studies showed inconclusive results in blood pressure changes after IV injection of PC/cholesterol liposome encapsulated ASODN. A significant decrease in blood pressure was observed after IV injection of cationic liposome-complexed ASODN, suggesting cationic liposome is more efficient in delivery ASODN than PC/cholesterol liposome.

The tissue distribution studies demonstrated that cationic liposome delivery increased specific tissue accumulation of ASODN, which may be a contributing factor leading to an increased inhibitory effect on blood pressure. One and 8 hours after IA administration, the liver had the highest accumulation of ASODN. About 30% of the injected cationic liposome complexed FITC-ASODN was shown to be present in the liver, probably due to the anatomical features of the reticuloendothelial system. After IV administration, more ASODN was seen to accumulate in the lungs, as expected. The accumulation in other organs, such as brain and heart, is minimal due to the continuous capillary endothelium present in the blood brain barrier and

in the heart muscle. At the 1 and 8 hour time point, the accumulation of ASODN in the kidney appears higher than that after CA/ASODN, probably because ASODNs were cleared by the liver faster than the CA/ASODN, which was larger and held in the liver tissues for a longer period of time. Twenty-four hours after injection, it appeared that the accumulation of CA/ASODN in the kidney is higher than after the naked ASODN. This could be a result of slow clearance of CA/ASODN from the RES compared to ASODN. The ASODN also were subjected to metabolism *in vivo*. The high intensity of fluorescent signal in the kidney 24 hours after injection might be a result of time-dependent elimination by the kidney. This could also be a possible reason for the lack of differences in accumulation between ASODN and CA/ASODN at 24 hours yet not the case at 1 and 8 hours.

Hepatic uptake and urinary excretion are the two major routes for the clearance of macromolecules, such as ASODN[147]. The distribution of macromolecules is basically restricted to the intravascular space immediately after injection, due to low capillary permeability in most organs. The ASODN distribution profile depends on the basic structural feature of capillaries which represent the major barrier between the circulation and the tissue cells and are diverse in porosity among organs[157]. The route that the molecules take to circulate in the body also determines their immediate disposition. Three types of capillary endothelium present in different tissues. Continuous

capillary, the most widely distributed, is found in cardiac muscle and constitutes the blood brain barrier. The transport pathway for molecules in this capillary is via small pores with estimated sizes of 6.7-8.0 nm and large pores of 20-28 nm. The fenestrated capillaries present in the intestinal mucosa and the glomerular and peritubular capillaries of the kidney, have openings with diameters of 40-60 nm. Discontinuous capillaries, which are found almost exclusively in liver, spleen and bone marrow, are characterized by the absence of a basement membrane and the presence of large endothelial gaps with diameters ranging between 100 to 1000 nm. The anatomical feature of the liver sinusoids allows macromolecules with sizes of 50-200 nm to pass out of the vascular space into the surrounding tissue easily[157].

For both IA and IV injection, hepatic uptake at the 1 and 8 hour time points is substantially higher due to the structure of the discontinuous endothelium of the liver, which brings the circulating molecules into free contact with the surface of the parenchymal cells. In the kidney, the glomerular capillary wall functions as a size and charge selective barrier. Macromolecules with a molecular weight of less than 50000, approximately 6 nm in diameter, are susceptible to glomerular filtration and are excreted into the urine easily. Larger molecules tend to have a higher renal accumulation due to both tubular reabsorption and uptake from the peritubular capillary side[147]. Little has

been reported on the effect of charge on the renal accumulation of ASODN. As shown in this study, at 1 and 8 hours after injection the ASODN seems to have a higher accumulation in the kidney than the complexed ASODN, while at 24 hours the higher distribution of complexed ASODN might be due to a time-dependent clearance.

In theory, antisense oligonucleotide blocks gene expression by activating RNase H with the subsequent degradation of the mRNA portion of the mRNA/ASODN hybrid. Compared with most pharmaceuticals which act on the end products of the gene expression, the proteins, ASODNs act on the earlier stage of the gene expression, at the mRNA level[75], making a more complete and long-lasting inhibitory effect possible. This *in vitro* studies showed that cationic liposome-complexed ASODN decreased AGT protein expression by 90% in hepatoma cell culture at a 1 μM concentration. In the *in vivo* situation, by optimizing pharmacokinetic parameters such as dosage and route of administration, substantial inhibition of the AGT gene expression also is expected *in vivo*.

CA/ASODN treatment resulted in a blood pressure decrease in SHR but not in WKY rats. The same effect was also observed after giving both strains of rats ACE inhibitors. The ACE inhibitor captopril caused a dose-dependent decrease in blood pressure with a maximal decrease of 38 mmHg in SHR, while no significant decrease in blood pressure was observed

in WKY[156]. These results suggest that overactivity of RAS could be a factor that leads to hypertension in the SHR strain.

A difference in blood pressure change after IA and IV injection was observed. However, the plasma AGT and Ang II level did not show significant differences after IA and IV administration. This may be explained by the similar distribution of CA/ASODN in the liver 24 hours after both routes of injection, since the liver is the only organ that provides the circulating AGT. This is also supported by the liver AGT mRNA level measured by Northern blot analysis, which showed a similar decrease of mRNA in the liver after either IA or IV injection.

The kidney and heart AGT mRNA were shown to be significantly lower 24 hours after IA than after IV administration, which might be a consequence of different ASODN uptake after different route of administration. Decreases in both the kidney and heart mRNA were greater after CA/ASODN than after naked ASODN treatment. These observed differences in AGT mRNA decreases in the kidney and the heart correlate with the difference in blood pressure decreases after IA and IV injection. This implies that the local RAS, possibly the kidney and the heart, might mediate the observed blood pressure changes. Decreases in AGT mRNA in local tissues, such as the kidney, may lead to a decrease in local RAS activity, which subsequently mediates tissue specific physiological effects on blood pressure.

The heart and kidney are two major organs in blood pressure regulation. Renin enzymatic activity was detected in the homogenate of whole heart[10]. The presence of both renin and AGT mRNA in the heart and the kidney was demonstrated using in situ hybridization. The coexpression of renin and the AGT gene makes the local de novo synthesis of angiotensin II possible and suggests the existence of a functioning local RAS. Angiotensin II, as a vasoactive hormone, has the effect of acutely increasing cardiac contractility or long term stimulation of cardiac hypertrophy. The kidney was the first organ for which a functioning tissue-specific RAS had been discussed. Co-expression of renin, AGT and angiotensin converting enzyme mRNA in the kidney support the existence of an intrarenal RAS, which functions to regulate renal sodium absorption and hemodynamics[154].

In summary, these results support the following conclusions: Cationic liposome complexed ASODN against AGT mRNA decreases blood pressure in SHR after IA and IV injection. Observed decreases in blood pressure were greater after IA rather than IV injection. The decrease in blood pressure was consistent with the decrease in AGT mRNA in the kidney and heart.

CHAPTER 5
DOSE-DEPENDENT PHYSIOLOGICAL EFFECTS OF CATIONIC LIPOSOME COMPLEXED ASODN IN SHR MODEL

Specific Aims

The specific aim of this part of the project is to study the dose-response effects of CA/ASODN (low:10 μg, medium:50 μg, high:500 μg) on blood pressure, plasma and tissue angiotensinogen levels and the water intake and urine output in the SHR model of hypertension in order to determine the role of kidney in ASODN mediated inhibition of hypertension.

Introduction

Blocking the renin angiotensin system by agents such as angiotensin converting enzyme inhibitors, renin inhibitors and angiotensin II receptor antagonists is one of the most important therapeutic options for treating hypertension[59]. Renin inhibitors have been shown to decrease blood pressure, angiotensin II and aldosterone levels in a dose-dependent manner[161,162]. A study conducted on essential hypertension in human subjects showed that the renin inhibitor, FK906, at doses of 25 mg, 50 mg, and 100 mg decreased blood pressures from a baseline level of 169±3/97±1 mmHg, to 153±5/87±3, 142 ±5/78±3 and 137±10/77±8 mmHg, respectively. The hypotensive

effects of FK906 did not correlate with baseline plasma renin activity, suggesting that the suppression of the non-circulating tissue renin-angiotensin system may account for the hypotensive effect[163]. Studies with another renin inhibitor, Remikiren, showed it decreased blood pressure in essential hypertension patients with concomitant increases in renal blood flow and sodium excretion[164]. An angiotensin II type I receptor antagonist, Losartan also has been shown to induce a dose-dependent inhibition of the pressor effect of angiotensin II. At a 100 mg or 150 mg dose, losartan decreases systolic blood pressure about 15 mmHg and 20 mmHg, respectively[165].

However, these agents, given alone or in combination with other drugs, control blood pressure in 60-80% of hypertensive patients[166]. In addition, all current drug therapies suffer from various problems, for example, angiotensin converting enzyme inhibitors, one of the most widely used agents that inhibit the renin angiotensin systems, are not specific for the RAS because they also inhibit kininase II, which degrades bradykinin. Increased levels of bradykinin cause undesirable side effects, such as dry cough, angioedema and urticaria[158]. Renin inhibitors, even though more specific, are limited by insufficient bioavailability [159], and angiotensin antagonists work well only on patients with high circulating angiotensin II[59]. Therefore, alternative routes to block the RAS may have potential advantages. Angiotensinogen, the precursor of the

RAS, has been suggested as an important determinant of blood pressure and electrolyte homeostasis[37]. Blocking angiotensinogen synthesis using antisense techniques has proved to be effective in decreasing blood pressure in the SHR model[64,65,66,68,160]. Antisense as a therapeutic option has the advantages of providing specific and long term pharmacological effects.

In a previous study, it was demonstrated that intra-arterially administered cationic liposome-complexed ASODN, at a dose of 50 μg(0.2 μg/kgBW), decreased blood pressure in SHR approximately 23 mmHg within 24 hours of administration [68]. Concomitant decreases in plasma AGT, angiotensin II, and tissue AGT mRNA were also observed. The decrease in blood pressure was correlated with a decrease in kidney AGT mRNA levels, rather than plasma AGT protein levels, suggesting that the observed blood pressure decreases may be mediated by the actions of the local renal tissue RAS.

Results from previous *in vitro* studies demonstrated that the cellular uptake of cationic liposome-complexed ASODN is dose-dependent[64]. Additionally, CA/ASODN decreased AGT mRNA and protein in a dose-dependent manner in Hepatoma cell culture. In an *in vivo* situation, angiotensinogen functions as the substrate of renin, and a substantial decrease in AGT level is required to lead to the decrease in activity of the RAS. High doses of CA/ASODN may cause depletion of the AGT pool in the body, which then decreases the activity of the

RAS, and subsequently increases the extent and duration of the antihypertensive effects.

One of the effects of angiotensin II is to stimulate the release of aldosterone from the adrenal cortex. Aldosterone acts on the renal distal tubule to increase the reabsorption of water and sodium and the excretion of potassium[3]. Blocking AGT expression should subsequently decrease angiotensin II levels and aldosterone release and reverse their effects. Angiotensin II also has a direct effect on the kidney by regulating vasoconstriction, renal blood flow, glomerular filtration rate, and sodium water excretion[165].

In this study, we measured the effect of low(10 μg), medium(50 μg), and high(500 μg) doses of CA/ASODN on blood pressure, plasma AGT, angiotensin II level and AGT mRNA levels in the heart, liver, kidney and its effects on plasma aldosterone, urinary sodium and potassium excretion, water intake and urine output. These results may provide information regarding the potential of ASODN as a therapeutic agent and on renal mechanisms of blood pressure regulation.

Material and Methods

To determine the dose-dependent effect of CA/ASODN on the level and duration of blood pressure changes, carotid artery catheterization was performed in SHRs. Animals were allowed to recover for 24 hours after surgery, then baseline blood pressures were measured using the tail cuff method. After

this, one of three doses of CA/ASODN, 10 μg(low),50 μg (medium) or 500 μg (high)(n=6), was injected via the carotid artery. A single dose of 500 μg of cationic liposome-complexed scrambled ODN was also administered as a control to determine the specificity of the antisense effect. Blood pressure was measured using the tail cuff method for 30 minutes at the same time every day for 5 days. During the week of study, rats were housed in the metabolic cages and their water intake and urine output was measured daily.

In a subsequent experiment, the dose-dependent effect of CA/ASODN on plasma AGT, Ang II, and aldosterone levels every 24 hours for 5 days was determined after a single injection. Carotid artery catheterization was performed in SHRs, and after a 24-hour recovery, either 10 μg, 50 μg or a 500 μg of CA/ASODN(n=3) was injected via the carotid artery. Again CA/ScrODN was used as a control(n=3). Groups of rats were sacrificed 24 hours after injection and plasma samples, liver, kidney and heart tissues were collected. Plasma AGT [124], angiotensin II(RK-A22, Alpco, Windham, NH), and aldosterone levels(Diagnostic Systems Laboratories, Inc. Texas) were measured using radioimmunoassay.

The dose-dependent effect of each dose of CA/ASODN on tissue AGT mRNA also was measured. Total mRNA was extracted from liver, kidney, and heart tissues. Messenger RNA was reverse transcripted to cDNA, and then subjected to amplification by polymerase chain reaction.

In the subsequent experiment, the dose effect of CA/ASODN on urinary sodium/potassium excretion at 24 hour intervals for 5 days after treatment was determined. The collected urine was centrifuged, and 200 µl of the supernatant was aspirated. Urinary sodium and potassium concentration was measured using NOVA 1+1 Automated Sodium/Potassium Analyzer (NOVA Biomedical, Massachusetts). The cumulative sodium and potassium excretion was calculated as the sodium and potassium concentration multiplied by the urine output.

Statistical analysis was performed by two way ANOVA for treatment effect. Data for individual time points were analyzed using the Students independent T-test. Significance was at the 95% confidence limit.

Results

Figure 5-1 demonstrates the dose-dependent effect of CA/ASODN on the blood pressure of SHR. The control SHRs showed a baseline level of 176±6 mmHg. At the 24 hour time point, a slight increase in blood pressure(183±6 mmHg) was observed. SHRs treated with the low dose of CA/ASODN showed blood pressure fluctuations within the normal range for the 5 days of measurement. SHRs treated with the medium dose of CA/ASODN showed a significant decrease in blood pressure on day 1(145±8 mmHg)($P<0.05$) and day 2 (151±4 mmHg)($P<0.05$) compared with the baseline level (168±6 mmHg) prior to administration. More pronounced decreases in blood pressure

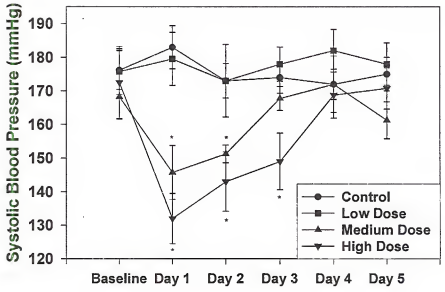


Figure 5-1. Dose-response effects of cationic liposome-complexed ASODN (CA/ASODN) on systolic blood pressure in SHR. SHRs were injected with either low(10 μg), medium(50 μg), high(500 μg) dose of CA/ASODN, or 500 μg CA/ScrODN intraarterially. Systolic blood pressures were monitored by tail-cuff every 24 hours for 5 days. Data represent mean ± standard error (n=4-6). (*) significant different from the baseline level ($P<0.05$).

were observed after the high dose treatment. Blood pressure was reduced from baseline levels of 172±10 mmHg to 132±8 mmHg on day 1($P<0.05$), 143±8 mmHg($P<0.05$) on day 2, and 149±8 mmHg($P<0.05$) on day 3. Blood pressures started to recover towards baseline levels on day 4(168±7 mmHg)(Figure 5-1).

CA/ASODN treatment also caused a dose-dependent decrease in plasma AGT. At the medium dose of CA/ASODN, the plasma AGT level decreased from a baseline level of 113±7 ng/ml to 57±6 ng/ml($P<0.05$) on day 1, to 62±11 ng/ml on day 2, and 88±7 ng/ml($P<0.05$) on day 3. Plasma AGT concentrations completely recovered to baseline levels on day 4. At the high dose of CA/ASODN, the decrease in plasma AGT levels was greater than of the medium dose and lasted for a longer period. The baseline levels of plasma AGT were 110±8 ng/ml, which was similar to the baseline values for the other experimental groups. This level was reduced to 45±8 ng/ml on day 1, 51±5 ng/ml on day 2($P<0.05$), 58±7 ng/ml on day 3, and 84±6 ng/ml on day 4($P<0.05$) (Figure 5-2). Plasma Ang II levels showed a similar decreasing trend with CA/ASODN treatment. After treatment with the medium dose of CA/ASODN, Ang II decreased from 217±13 ng/ml to 74±15 ng/ml on day 1, and to 92±11 ng/ml on day 2. At the high dose, Ang II levels decreased more profoundly, from 198±31 ng/ml to 37±5 ng/ml on day 1, 74±15 ng/ml on day 2, and 94±12 ng/ml on day 3($P<0.05$). The reduction of plasma Ang II lasted for 3 days and started to recover towards baseline on day4(Figure 5-3).

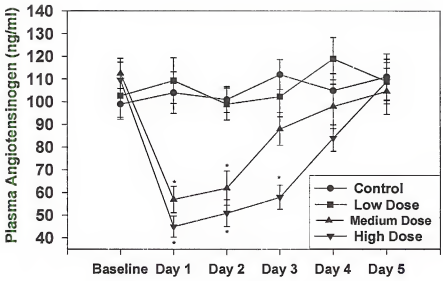


Figure 5-2. Dose-response effects of CA/ASODN on plasma AGT levels in SHR. SHRs were injected with one of three doses of CA/ASODN, low(10 µg), medium(50 µg), high(500 µg) or 500 µg CA/ScrODN intraarterially. Plasma angiotensinogen levels were measured by radioimmunoassay every 24 hours for 5 days. Data represent mean ± standard error(n=3). (*) significant different from the baseline levels($P<0.05$).

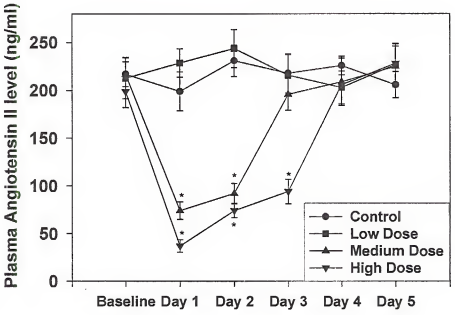
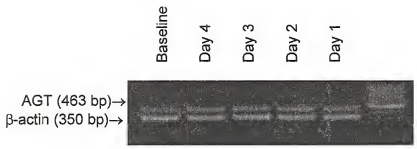


Figure 5-3. Dose-response effects CA/ASODN on plasma angiotensin II levels in SHR. SHRs were injected with one of three doses of CA/ASODN, low(10 μg), medium(50 μg), high (500 μg) or 500 μg CA/ScrODN intraarterially. Plasma angiotensin II levels were measured by radioimmunoassay every 24 hours for 5 days. Data represent mean ± standard error(n=3). (*) significant different from the baseline levels ($P<0.05$).



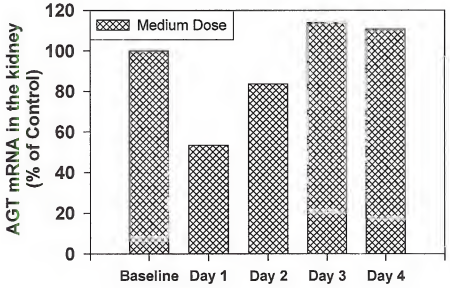
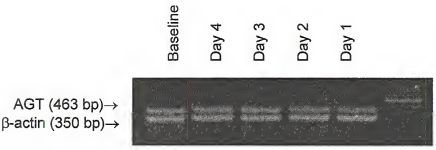


Figure 5-4. Dose-response effects of medium dose CA/ASODN on kidney AGT mRNA expression in SHR. SHRs were injected with 50 μg of CA/ASODN intra-arterially. AGT mRNA expression in the kidney was measured by RT-PCR. Upper: Result from the RT-PCR. AGT:463 bp, β-actin: 350 bp; Lower: Ratio of the intensity of AGT to control β-actin bands. Results were consistent for three measurements.



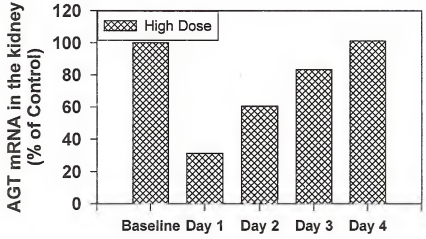
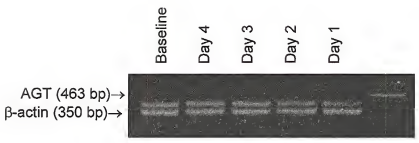


Figure 5-5. Dose-response effects of high dose CA/ASODN on kidney AGT mRNA expression in SHR. SHRs were injected with 500 μg of CA/ASODN intra-arterially. AGT mRNA expression in the kidney was measured by RT-PCR. Upper: Result from the RT-PCR. AGT:463 bp, β-actin: 350 bp; Lower: Ratio of the intensity of AGT to control β-actin bands. Results were consistent for three measurements.



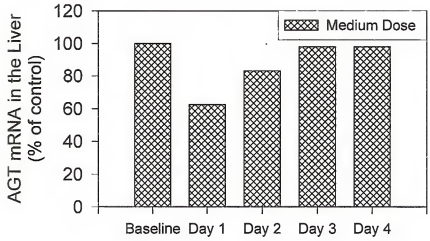


Figure 5-6. Dose-response effects of medium dose CA/ASODN on liver AGT mRNA expression in SHR. SHRs were injected with 50 μg of CA/ASODN. AGT mRNA expression in the liver were measured by RT-PCR. Upper: Result from the RT-PCR. AGT:463 bp, β-actin:350 bp; Lower: Ratio of the intensity of AGT to control β-actin bands(n=3). Results were consistent for three measurements.

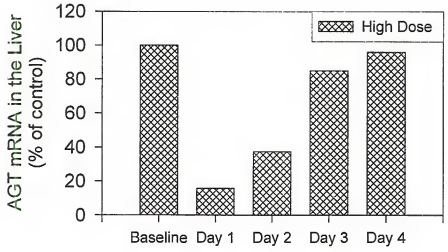
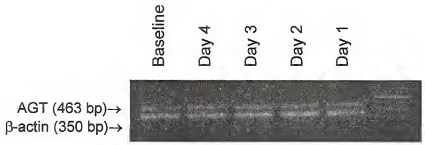


Figure 5-7. Dose-response effects of high dose CA/ASODN on liver AGT mRNA expression in SHR. SHRs were injected with 500 μg of CA/ASODN. AGT mRNA expression in the liver were measured by RT-PCR. Upper: Result from the RT-PCR. AGT:463 bp, β-actin:350 bp; Lower: Ratio of the intensity of AGT to control β-actin bands(n=3). Results were consistent for three measurements.



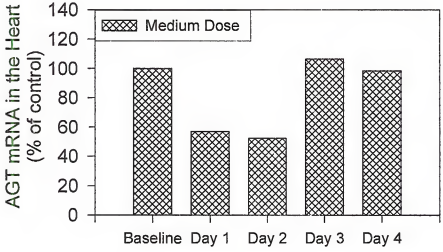
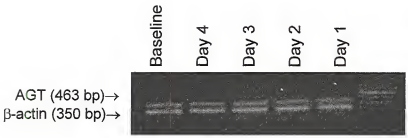


Figure 5-8. Dose-response effects of medium dose CA/ASODN on heart AGT mRNA expression in SHR. SHRs were injected with 500 μg dose of CA/ASODN. AGT mRNA expressions in the heart were measured by RT-PCR. Upper: Result from the RT-PCR. AGT:463 bp, β-actin:350 bp; Lower: Ratios of the intensity of AGT band to control β-actin(n=3). Results were consistent for three measurements.



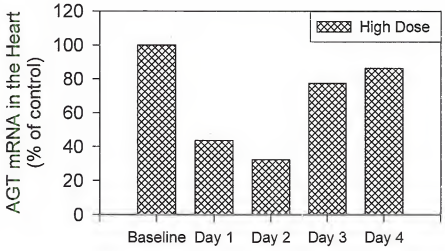


Figure 5-9. Dose-response effects of high dose CA/ASODN on heart AGT mRNA expression in SHR. SHRs were injected with 500 μg of CA/ASODN. AGT mRNA expressions in the heart were measured by RT-PCR. Upper: Result from the RT-PCR. AGT:463 bp, β-actin:350 bp; Lower: Ratios of the intensity of AGT band to control β-actin(n=3). Results were consistent for three measurements.

The dose dependent effect of CA/ASODN on tissue AGT mRNA also was studied. AGT mRNAs were quantified by the RT-PCR method. Figure 5-4, 5-5 and 5-6 show the ratios of AGT mRNA to control β-actin mRNA. The figures demonstrate that kidney AGT mRNA decreased 70% on day 1 after the high dose treatment, with full recovery occurring on day 4. With the medium dose, AGT mRNA decreased 50% on day 1 and recovered by day 3(Figure 5-4). In the liver, AGT mRNA was decreased 90% and 50% on the first day after the high and medium dose of CA/ASODN treatments, respectively. This reduction lasted about 2 days(Figure 5-5). The high dose of CA/ASODN treatment also induced a significant decrease of AGT mRNA in the heart, as shown in Figure 5-6. Greater decreases of AGT mRNA were observed after the high dose CA/ASODN treatment than after the medium dose.

Table 5-1 presents the water intake and urine output of SHR's after CA/ASODN treatment. Since the rats were taken from metabolic cages for 3-5 hours every day for blood pressure measurement, the values reflect water intake and urine output during an 18-20 hour period rather than 24 hours. The water intake fluctuated daily and no significant difference could be attributed to the CA/ASODN treatment when compared with controls or even within groups. However, urine volumes were significantly increased ($P<0.05$) after CA/ASODN treatment. With the medium dose, urine volumes increased to 11.2±1.9 ml ($P<0.05$) on day 1, 12.4±3.3 ml on day 2 ($P<0.05$) compared with the baseline urine output of

Table 5-1. Dose-response effects of CA/ASODN on water intake and urine output in SHR. SHRs were injected with medium (50 µg) or high dose(500 µg) of CA/ASODN intra-arterially. Urine output and drinking volume were measured daily for 5 days (n=4-6). BL: baseline. P<0.05(*).

	Water intake ± S.E. (ml/24 h)(n=4-6)	Urine output ± S.E. (ml/24 h)(n=4-6)
Control(BL)	31.8±2.7	9.6±2.2
Day 1	26.4±3.7	7.9±1.8
Day 2	28.3±2.6	9.0±1.5
Day 3	27.3±3.9	10.1±2.2
Day 4	24.2±4.1	9.7±1.3
Day 5	26.8±2.1	11.3±0.9
Medium dose(BL)	32.2±1.9	9.1±1.3
Day 1	29.2±3.1	11.2±1.9*
Day 2	35.1±3.7	12.4±3.3*
Day 3	26.7±2.8	10.2±2.9
Day 4	31.3±1.8	9.0±1.7
Day 5	27.7±2.7	9.5±0.5
High dose(BL)	26.5±2.5	8.3±0.9
Day 1	28.3±2.2	12.8±1.1*
Day 2	31.7±3.7	11.7±1.6*
Day 3	29.7±1.7	11.3±0.6*
Day 4	33.3±5.1	8.5±0.6
Day 5	25.7±3.2	8.7±0.7

Table 5-2. Dose-response effects of CA/ASODN on plasma aldosterone levels in SHR. SHRs were injected with medium (50 μg) or high dose(500 μg) of CA/ASODN intra-arterially. Plasma aldosterone levels were measured by radioimmunoassay every 24 hours for 5 days(n=3). $P<0.05$(*)

	Aldosterone ± S.E.(ng/ml)
Control(baseline)	722±52
day 1	750±34
day 2	700±68
day 3	712±56
day 4	745±71
day 5	717±44
Medium dose(baseline)	788±36
day 1	620±26*
day 2	640±46*
day 3	594±44*
day 4	741±29
day 5	765±51
High dose(baseline)	774±37
day 1	379±19*
day 2	358±23*
day 3	333±15*
day 4	678±39
day 5	773±51

9.1±1.3 ml. At the high dose, this significant increase in urine volume was greater and more prolonged: 12.8±1.1 ml on day 1, 11.7±1.6 ml on day 2, and 11.3±0.6 ml on day 3($P<0.01$), compared with a baseline level of 8.3±0.9 ml.

Plasma aldosterone levels in response to CA/ASODN treatment were also determined in the SHRs. Significant decreases in aldosterone were observed after both the medium and high dose treatments. At medium doses, aldosterone levels dropped from a baseline level of 788±36 ng/ml to 620± 26 ng/ml on day 1, 640±46 ng/ml on day 2, and 594±44 ng/ml($P<0.01$) on day 3. Aldosterone values were normalized by day 4. After the high dose of CA/ASODN, the reduction in aldosterone levels was more impressive. From a baseline value of 774±37 ng/ml, aldosterone level was reduced to 379± 19 ng/ml on day 1, 358±23 ng/ml on day 2, and 333±15 ng/ml on day 3($P<0.01$). Aldosterone values were not altered in SHRs treated with control ScrODN (Table 5-2). The observed decreases in aldosterone levels were consistent with urinary sodium and potassium excretion. Prior to CA/ASODN treatment, urinary sodium and potassium excretion was similar in all groups during the 20 hour measurement period. There were no changes observed in the control group over the 5 day measurement. However, a dose-response like effect on sodium and potassium excretion was observed in the CA/ASODN treatment groups. An increase in the cumulative sodium excretion with a concomitant decrease in potassium excretion

Table 5-3. Dose-response effects of CA/ASODN on 24-hour urinary sodium and potassium excretion in SHR. SHRs were injected with medium (50 µg) and high (500 µg) doses of CA/ASODN intra-arterially. The sodium and potassium concentrations in the urine were measured and total sodium and potassium excretions were calculated as the concentration multiplied by the urine volume (n=4-6). P<0.05(*)

	Sodium ± S.E. (mmol/24 h)(n=4-6)	Potassium ± S.E. (mmol/24 h)(n=4-6)
Control (BL)	1.82±0.05	1.37±0.03
day 1	1.78±0.03	1.36±0.03
day 2	1.88±0.08	1.41±0.03
day 3	1.85±0.11	1.39±0.04
day 4	1.83±0.06	1.38±0.02
day 5	1.79±0.04	1.41±0.04
Medium dose(BL)	1.80±0.09	1.32±0.05
Day 1	2.02±0.10*	1.13±0.05*
Day 2	1.96±0.10*	1.15±0.08*
Day 3	1.80±0.07	1.35±0.06
Day 4	1.77±0.06	1.34±0.09
Day 5	1.81±0.12	1.4±0.09
High dose(BL)	1.80±0.11	1.33±0.06
Day 1	2.21±0.10*	1.07±0.07*
day 2	2.14±0.18*	1.10±0.09*
day 3	1.98±0.11*	1.25±0.06*
day 4	1.84±0.06	1.40±0.07
day 5	1.77±0.08	1.47±0.08

was observed. With the medium dose of CA/ASODN treatment, sodium excretion increased from a baseline value of 1.80± 0.09 mmol to 2.02±0.1 mmol on day 1, and 1.96±0.1 mmol on day 2, while potassium excretion decreased from a baseline level of 1.32±0.05 mmol to 1.13±0.05 mmol on day 1, and 1.15 ±0.08 mmol on day 2($P<0.01$). After the high dose, sodium excretion increased from a baseline level of 1.80±0.11 mmol to 2.21±0.1 mmol on day 1, 2.14±0.18 mmol on day 2, and 1.98 ±0.08 mmol on day 3. Potassium excretion decreased from a baseline level of 1.33±0.06 mmol to 1.07±0.07 mmol on day 1, 1.10±0.09 mmol on day 2, and 1.25±0.06 mmol on day 3($P<0.01$) (Table 5-3).

Discussion

The results of this study demonstrate that intra-arterial injection of cationic liposome-complexed ASODN targeted against AGT mRNA decreased blood pressure in a dose-dependent manner. Blood pressure decreased significantly at both 50μg and 500μg doses in SHR, while injection of scrambled ODN- complex did not produce a similar effect, suggesting specificity of the antisense action at these concentrations. At the 500μg dose, a 40 mmHg decrease in systolic blood pressure was observed. The decrease in blood pressure lasted about 72 hours, compared with the medium dose treated SHRs, which showed a smaller decrease in blood pressure with a shorter duration of action.

Decreasing blood pressure in SHR via inhibition of the AGT gene expression using an antisense technique has been reported in the literature[66,68,160]. The antihypertensive effects differ based on routes of antisense administration and methods of hepatocyte delivery. Tomita, et al. transfected antisense against AGT mRNA via the hepatic portal vein using liposomes containing viral agglutinins. ASODN at a concentration of 5 μM decreased blood pressure from 172 mmHg to 154 mmHg. The decrease in blood pressure lasted for 3 days[66]. Makino, et al injected ASODN against rat angiotensinogen coupled to an asialoglycoprotein carrier molecule via the tail vein. They observed a blood pressure decrease from 201 mmHg to 171 mmHg at a 50ug ASODN dose 24 hours after injection[160]. However, none of these methods were able to decrease blood pressure to normotensive levels.

In this study, it has been demonstrated that an intra-arterial injection of a 500μg dose of CA/ASODN was able to decrease blood pressure from a baseline of 172±10 mmHg to 132±8 mmHg after 24 hours. This single dose of CA/ASODN produced a hypotensive effect comparable to the antihypertensive effect produced by multiple dosings of other drugs working on the renin angiotensin system such as ACE inhibitors and renin inhibitors[164]. This suggests that antisense blocking the renin angiotensin system may have potential as an antihypertensive agent with longer duration. These results also support the fact that AGT is important in blood pressure regulation.

The observed antihypertensive effect was dose dependent. A more pronounced decrease in blood pressure was observed after high dose treatment compared with that of the medium dose. Our previous in vitro study showed that the uptake of CA/ASODN reached a plateau at CA/ASODN concentration at about 1 μM in cell culture. In an *in vivo* situation, increasing the dose should increase the plasma concentration and then lead to a more effective intracellular delivery of ASODN, subsequently increasing the extent and duration of the biological effect. At these concentrations lesser cellular toxicity has been shown after *in vitro* studies.

To elucidate the underlying mechanisms for the blood pressure decrease, plasma AGT, angiotensin II levels and tissue AGT mRNA expression are subsequently measured. A significant, dose-dependent decrease in plasma AGT and angiotensin II was observed. The duration of the plasma AGT and angiotensin II decreases was consistent with that of the decrease in blood pressure, suggesting a possible role of plasma RAS in the blood pressure decrease at these doses.

In heart, kidney and liver, a dose-dependent decrease in AGT mRNA was observed 1-3 days after treatment. The decrease in AGT mRNA in the liver lasted for 3 days, which corresponds to observed plasma AGT levels, possibly because liver is the primary organ that contributes to the circulating AGT. The decrease in AGT mRNA was more pronounced at the high dose, suggesting that an increase in

tissue and cellular uptake may produce a greater inhibition of AGT gene expression.

At a medium or high dose CA/ASODN induced increased urine output, suggesting a role of increased fluid excretion for the observed decrease in blood pressure. To specify the mechanism for this apparent diuretic or natriuretic effect, plasma aldosterone levels and urinary sodium and potassium output are subsequently measured. Aldosterone was secreted by the zona glomerulosa of the adrenal cortex[167]. All of the components of the renin angiotensin system have been identified in the adrenal cortex[168]. The mechanism by which the renin-angiotensin system acts within the adrenal is not known. One possibility is that all of the reactions take place within the zona glomerulosa cells and the final product angiotensin II stimulates aldosterone production. A 50% decrease in plasma aldosterone was observed after the high dose treatment. This decrease in aldosterone also might be a direct response of the decrease in angiotensin II level produced by ASODN treatment. A decrease in angiotensin II inhibits the release of aldosterone from the adrenal cortex, with a subsequent increase in water and sodium reabsorption from the distal tubule of the kidney. The results of 24-hour cumulative sodium and potassium excretion supports the role played by the aldosterone; an increase in sodium excretion with a concomitant decrease in potassium excretion was observed. Increased water

excretion could also be mediated by the direct effect of angiotensin II on the kidney. Additionally, an increase in water excretion could also result from the action of antidiuretic hormone(ADH). Angiotensin II is known to stimulate the release of ADH from the posterior pituitary. ADH increases reabsorption of water by the distal tubule and collecting ducts, thus increasing fluid volume [169]. Since Ang II increases ADH release, the decreases in Ang II levels may reduce ADH levels, which in turn could result in greater urine loss. The water intake did not change significantly after the CA/ASODN treatment, suggesting that increased urine output is not strictly a coupled reaction to fluid intake.

In summary, these results support the following conclusions. First, intra-arterial injection of CA/ASODN decreased blood pressure in a dose-dependent manner, with a 40±3 mmHg decrease in blood pressure after a 500 ug dose of ASODN treatment. Second, plasma AGT, angiotensin II, and tissue AGT mRNA also showed dose-dependent decreases. Third, the treatment also resulted in a decrease in plasma aldosterone levels, with a concomitant increase in sodium excretion and a decrease in potassium excretion. These results suggest that an aldosterone mediated kidney effect be involved in the observed blood pressure decrease.

CHAPTER 6
DISCUSSION AND SUMMARY

The unique property of antisense oligonucleotides, which intervenes and inhibits a gene expression with high specificity, provides a great advantage for use as both a therapeutic agent and a research tool. Although various problems are not completely solved, certain factors regarding their application, such as stability and delivery, have improved over the years. More than a dozen ASODNs designated for both topical use and in vivo applications are now undergoing clinical trials[78].

In these studies, it has been proposed to apply ASODN technology to study the mechanism of essential hypertension in the SHR model and to study the potential of development of an ASODN as antihypertensive agent with long lasting therapeutic effects. In the first part of this study, the efficiency of cationic liposomes composed of DDAB and DOPE, as a delivery system for the cellular delivery of ASODN in hepatoma cell culture was evaluated. The results demonstrated that cationic liposome-complexation increased the rate and extent of cellular delivery of ASODN in a dose dependent manner. The increased delivery subsequently led to increased inhibition of targeted gene expression. Both AGT

mRNA and protein expression decreased in a dose-dependent manner.

The composition of the cationic lipids and ASODN affects its interactions with cells. The cellular uptake of CA/ASODN changed dramatically with different lipids/ASODN ratios, possibly due to such factors as size, charge, and membrane interaction. The overall positive charge of the complex facilitates its adsorption onto the cell surface and subsequent intracellular ASODN delivery. Cationic liposomes also appeared to assist the escape of the ASODN from intracellular compartments.

A second specific goal was to study the effects of the route of administration of CA/ASODN on blood pressure and tissue distribution of ASODN in SHR. Blood pressures decreased more profoundly after intraarterial administration than after intravenous administration. Plasma AGT and angiotensin II levels decreased similarly after both IA and IV injection. However, AGT mRNA in the heart and the kidney tissues showed a greater decrease after IA than after IV injection, suggesting that a tissue specific effect may be involved. Our tissue distribution studies showed that cationic liposomes increased the disposition of ASODN in the measured tissues.

In specific aim three, the dose-dependent effects of CA/ASODN on blood pressure, plasma and tissue RAS in SHR was studied. Blood pressure, plasma AGT, angiotensin II and tissue AGT mRNA decreased in a dose-dependent manner. As

expected, these effects lasted longer at the higher dose. Plasma aldosterone levels also decreased, with a concomitant increase in urinary output, with an increase in sodium excretion and a decrease in potassium excretion. This suggests that the decrease in blood pressure may be the result of kidney effects mediated by aldosterone. The possible mechanisms underlying the observed ASODN mediated blood pressure decrease is summarized in Figure 6-1.

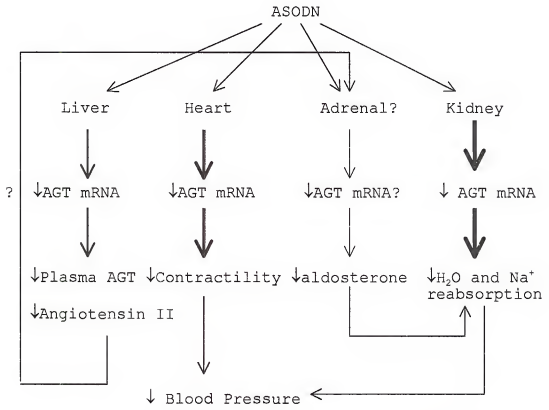


Figure 6-1. Summary of possible mechanisms for the observed ASODN mediated blood pressure decrease.

From linkage genetic studies, AGT has been indicated to be one of the candidate genes involved in the pathogenesis

of hypertension. These findings suggest that both circulating and tissue AGT, such as in kidney and heart, play an important role in the pathogenesis of hypertension in the SHR. Even though feedback mechanisms may function to regulate blood pressure, it appears that inhibition of AGT gene expression by ASODN was effective in decreasing high blood pressure. The results also demonstrated that a single dose of CA/ASODN produced a long lasting hypotensive effect compared with other antihypertensive agents. All these results support the fact that ASODN targeted against AGT could potentially be used as a therapeutic agent for the treatment of hypertension.

Successful decreases in blood pressure may be mediated by effective tissue targeting of ASODN. The results of this study support the finding that the cationic liposome approach is an effective method for delivering the ASODN to the liver. Specific gene targeting to the liver has been reported in the literature. Wu et al.[170] developed asialogly-coprotein-polylysine-DNA conjugates to target asialoglyco-protein receptors, which are uniquely expressed by hepato-cytes. Successful hepatic expression of the exogenous gene after IV injection was observed. Zhu et al.[171], employed a cationic liposome approach to deliver a model plasmid. They obtained variable gene expression in tissues in an *in vivo* study. In a more recent study, Makino et al.[160], reported that an intravenous injection of asialoglycoprotein-poly-L)lysine-antisense complex atrgeted

to AGT reduced plasma AGT levels and hepatic AGT mRNA as well as systolic blood pressure in SHR. The effects lasted for five days, whereas the control sense complex did not produce similar effects.

The routes and doses of CA/ASODN administration also were suggested to be important factors. These results showed that at a 500 ug dose, blood pressure decreased more after IA than after IV injection. A 40 mmHg decrease in blood pressures was observed after the 500ug IA injection of CA/ASODN. IV injection of a 50 ug dose produced a less but significant decrease in blood pressure. The effect of a high dose of CA/ASODN on blood pressure when administered IV was not determined in this study. However, a significant decrease in blood pressures after IV injection of this dose is expected. The IV route of administration is a more practical approach for its application than IA route, which is rarely used in the clinic setting. An in vitro study showed that the cellular uptake of CA/ASODN is dose-dependent. In an in vivo situation, a dose-dependent tissue uptake of ASODN also is expected. Increasing the dose may increase the amount of ASODN that get to the target site. Our tissue distribution studies showed that a substantial amount of CA/ASODN accumulates in the lung after IV injection than after IA injection. This may decrease the dose availability reaching the liver or kidney. By increasing the dose to 500 μg, and using IV administration,

more CA/ASODN may reach the target site such as observed via the IA route of administration.

In summary, these studies support the following conclusions. First, cationic liposomes composed of DDAB and DOPE are more effective in delivering ASODN in hepatoma cell culture and in a rat model of hypertension than previously used phosphatidylcholine cholesterol liposomes. Second, ASODNs targeted to AGT mRNA decrease blood pressure in SHR hypertension model after IA and IV injection, with the hypotensive effects being more pronounced after IA administration. Cationic liposome complexation potentiates this effect and the observed decreases in blood pressure correlate with the decreases in AGT mRNA expression in the kidney. Third, CA/ASODN decreases blood pressure, plasma AGT, angiotensin II and AGT mRNA in a dose-dependent manner. The plasma aldosterone levels also demonstrate a dose-dependent decrease, with concomitant increases in urinary sodium excretion and decreases in urinary potassium excretion. Collectively, these results suggest that the blood pressure changes observed with CA/ASODN treatment may be mediated via tissue effects, in particular, the kidney.

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